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<b>(21) International Application Number:</b> PCT/US99/31041 <b>(22) International Filing Date:</b> 29 December 1999 (29.12.99)  <b>(30) Priority Data:</b> 60/114,580 31 December 1998 (31.12.98) US  <b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application</b> US Not furnished (CIP) Filed on Not furnished  <b>(71) Applicants (for all designated States except US):</b> MILLENNIUM PHARMACEUTICALS, INC. [US/US]; 75 Sidney Street, Cambridge, MA 02139 (US). PFIZER LIMITED [GB/GB]; Ramgate Road, Sandwich, Kent CT13 9NJ (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> DORR, Patrick, K. [GB/GB]; 28 Pretoria Road, Canterbury, Kent CT1 1QL (GB). PARKINSON, Tanya [GB/GB]; Rose Villa, Chapel Lane, Ashley, Dover, Kent CT15 5HX (GB). BULAWA, Christine [US/US]; 11 Grandview Road, Arlington, MA 02174 (US).		<b>(74) Agent:</b> FASSE, J., Peter; Fish & Richardson, P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).  <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i> <i>With an indication in relation to deposited biological material furnished under Rule 13bis separately from the description.</i>
<b>(54) Title:</b> A. FUMIGATUS ACETYL COENZYME-A CARBOXYLASE GENES AND POLYPEPTIDES AND USES THEREOF  <b>(57) Abstract</b>  Disclosed are <i>Aspergillus fumigatus</i> coenzyme-A carboxylase genes and polypeptides and their use in identifying antifungal agents, for example.		

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A. FUMIGATUS ACETYL COENZYME A CARBOXYLASE  
GENES AND POLYPEPTIDES AND USES THEREOF

Cross-reference to Related Application

This application claims priority under 35 U.S.C. §119  
10 from USSN 60/114,580, filed December 31, 1998.

Field of the Invention

The invention relates to acetyl coenzyme A  
carboxylase genes of the fungus *Aspergillus fumigatus* and  
15 their use in identifying antifungal agents.

Background of the Invention

The enzyme acetyl coenzyme A carboxylase (ACCase) is  
responsible for synthesizing malonyl CoA from acetyl CoA.  
20 ACCase is essential for synthesis of fatty acids.

By way of background, the Fungi Kingdom consists of  
two divisions, the Eumycota and Myxomycota or the true fungi  
and slime molds, respectively. The true fungi are those  
species that are hyphal or are clearly related to species  
25 that are hyphal, possess cell walls throughout most or all of  
their life cycle, and are exclusively absorptive in their  
function. The slime molds are organisms that do not form  
hyphae, lack cell walls during the phase in which they obtain  
nutrients and grow and are capable of ingesting nutrients in  
30 particulate form by phagocytosis.

The two most important classes of true fungi in which  
most species produce motile cells, known as zoospores, are  
the Oomycetes, and the Chytridiomycetes. The fungi that lack  
zoospores are classified according to the sexual phase of the  
35 fungal life cycle. The sexual process leads to the  
production of characteristic spores in the different groups.  
The fungi that form zygospores are classified as Zygomycetes,

those that form ascospores are classified as Ascomycetes, and those forming basidiospores are classified as Basidiomycetes. There are also many species, recognizable as higher fungi through the presence of cell walls in their hyphae, that  
5 produce asexual spores but lack a sexual phase. These are known as Deuteromycetes, and details of their asexual sporulation are used to classify them. A representative member of the Deuteromycetes includes *Candida albicans*. These species are extensively reviewed in "The Fungi" (Ed M J  
10 Carlile and SC Watkinson 1994 Acad Press Ltd) and "The Growing Fungus" (Ed. NAR Gow and GM Gadd, 1995, Chapman and Hall).

Yeast are fungi that are normally unicellular and reproduce by budding although some will, under appropriate  
15 conditions, produce hyphae, just as some normally hyphal fungi may produce a yeast phase. The best known of all yeasts is *Saccharomyces cerevisiae*, which is a member of the Ascomycetes species. It is commonly regarded as a diploid yeast since mating usually soon follows ascospore  
20 germination. However, single cells can be used to establish permanently haploid cultures.

Fungal and other mycotic pathogens are responsible for a variety of diseases in humans, animals and plants. Fungal infection is also a significant problem in veterinary  
25 medicine. Some of the fungi that infect animals can be transmitted from animals to humans. Fungal infections or infestations are also a very serious problem in agriculture with fungicides being employed to protect vegetable and fruit and cereal crops. Fungal attack of wood products is also of  
30 major economic importance. Additional products that are susceptible to fungal infestation include textiles, plastics, paper and paint. Some of these fungal targets are extensively reviewed in WO 95/11969.

Statistics show that the incidence of fungal infections has doubled from the 1980's to the 1990's, and infections of the blood stream have increased fivefold with an observed mortality of 50% (Tally et al., 1997, Int. Conference  
5 Biotechnol Microb. Prods: Novel Pharmacol. Agrobiol. Activities, Williamsburg, VA Abstr S5 p19). These include those fungal infections, such as candidiasis, to which all individuals are susceptible, but also infections such as cryptococcosis and aspergillosis, which occur particularly in  
10 patients of compromised immune status.

By way of example, the yeast *Candida albicans* (*C. albicans*) has been shown to be one of the most pervasive fungal pathogens in humans. It has the capacity to opportunistically infect a diverse spectrum of compromised  
15 hosts, and to invade many diverse tissues in the human body. It can in many instances evade antibiotic treatment and the immune system. Although *Candida albicans* is a member of the normal flora of the mucous membranes in the respiratory, gastrointestinal, and female genital tracts, in such  
20 locations, it may gain dominance and be associated with pathologic conditions. Sometimes it produces progressive systematic disease in debilitated or immunosuppressed patients, particularly if cell-mediated immunity is impaired. Sepsis may occur in patients with compromised cellular  
25 immunity, e.g., those undergoing cancer chemotherapy or those with lymphoma, AIDS, or other conditions. *Candida* may produce bloodstream invasion, thrombophlebitis, endocarditis, or infection of the eyes and virtually any organ or tissue when introduced intravenously, e.g., via tubing, needles,  
30 narcotics abuse etc.

*Candida albicans* has been shown to be diploid with balanced lethals, and therefore probably does not go through a sexual phase or meiotic cycle. This yeast appears to be

able to spontaneously and reversibly switch at high frequency between at least seven general phenotypes. Switching has been shown to occur not only in standard laboratory strains, but also in strains isolated from the mouths of healthy  
5 individuals.

Nystatin, ketoconazole, and amphotericin B are drugs that have been used to treat oral and systemic *Candida* infections. However, orally administered nystatin is limited to treatment within the gut and is not applicable to systemic  
10 treatment. Some systemic infections are susceptible to treatment with ketoconazole or amphotericin B, but these drugs may not be effective in such treatment unless combined with additional drugs. Amphotericin B has a relatively narrow therapeutic index and numerous undesirable side  
15 effects, ranging from nausea and vomiting to kidney damage and toxicities occur even at therapeutic concentrations. While ketoconazole and other azole antifungals exhibit significantly lower toxicity, their mechanism of action, through inactivation of cytochrome P<sub>450</sub> prosthetic group in  
20 certain enzymes (some of which are found in humans) precludes use in patients that are simultaneously receiving other drugs that are metabolized by the body's cytochrome P<sub>450</sub> enzymes. These adverse effects mean that their use is generally limited to the treatment of topical or superficial  
25 infections. In addition, resistance to these compounds is emerging and may pose a serious problem in the future. The more recently developed triazole drugs, such as fluconazole, are believed by some to have fewer side effects but are not completely effective against all pathogens.

30 Invasive aspergillosis, caused by *Aspergillus fumigatus* (*A. fumigatus*) has also become an increasingly opportunistic infection. There has been a 14-fold increase in its incidence during the past 12 years as detected by autopsy,

and only two drugs are available that are effective in its treatment, neither of which is completely satisfactory. Amphotericin B needs to be given intravenously and has a number of toxic side effects. Itraconazole, which can be  
5 given orally is often prescribed imprudently, encouraging the emergence of resistant fungal strains (Dunn-Coleman and Prade, Nature Biotechnology, 1998, 16: 5). Resistance is also developing to synthetic azoles (such as fluconazole and flucytosine), and the natural polyenes (such as amphotericin  
10 B) are limited in use by their toxicity.

Fungicide resistance generally develops when a fungal cell or fungal population that originally was sensitive to a fungicide becomes less sensitive by heritable changes after a period of exposure to the fungicide.

15 In certain applications, such as agriculture, it is possible to combat resistance through alteration of fungicides or the use of fungicide mixtures. To prevent or delay the build up of a resistant pathogen population, different agents that are effective against a particular  
20 disease must be available. One way of increasing the number of available agents is to search for new site-specific inhibitors.

Consequently, antifungal drug discovery efforts have been directed at components of the fungal cell or its  
25 metabolism that are unique to fungi, and hence might be used as therapeutic targets of new agents which act on the fungal pathogen without undue toxicity to host cells. Such potential targets include enzymes critical to fungal cell wall assembly (US-A-5194600) as well as topoisomerases  
30 (enzymes required for replication of fungal DNA). Two semisynthetic antifungal agents such as the echinocandins and the related pneumocandins are in late stage clinical trials. Both are cyclic lipopeptides produced by fungi that non-

competitively inhibit (1,3)-glucan synthase and thus interfere with the biosynthesis of the fungal cell wall. These clinical candidates are generally more water-soluble, have improved pharmacokinetics and broader antifungal spectra  
5 than their natural parent compounds and have activity spectra that include many *Candida* species, including *Candida albicans*, and *Aspergilli*.

Because no single approach may be effective against all fungal pathogens, however, and because of the possibility of  
10 developed resistance to previously effective antifungal compounds, there remains a need for new antifungal agents with novel mechanisms of action and improved or different activity profiles. There is also a need for agents which are active against fungi but are not toxic to mammalian cells, as  
15 toxicity to mammalian cells can lead to a low therapeutic index and undesirable side effects in the host (e.g., patient). An important aspect of meeting this need is the selection of an appropriate component of fungal structure or metabolism as a therapeutic target.

20 Even after a particular intracellular target is selected, the means by which new antifungal agents are identified pose certain challenges. Despite the increased use of rational drug design, a preferred method continues to be the mass screening of compound "libraries" for active  
25 agents by exposing cultures of fungal pathogens to the test compounds and assaying for inhibition of growth. In testing thousands or tens of thousands of compounds, however, a correspondingly large number of fungal cultures must be grown over time periods which are relatively long compared to most  
30 bacterial culture times. Moreover, a compound which is found to inhibit fungal growth in culture may be acting not on the desired target but on a different, less unique fungal component, with the result that the compound may act against



host cells as well and thereby produce unacceptable side effects. Consequently, there is a need for an assay or screening methods which more specifically identifies those agents that are active against a certain intracellular  
5 target. Additionally, there is a need for assay methods having greater throughput, that is, assay methods which reduce the time and materials needed to test each compound of interest.

Although cyclic lipopeptides produced by fungi are in  
10 late-stage clinical trials as potential anti-fungal agents, the lipid biosynthesis and degradation pathways have been only sparingly investigated in fungi. This area is reviewed by Weete (1980 Lipid Biochemistry of Fungi and Other  
Organisms, Plenum New York) and Chopra and Khuller (1984 Crit  
15 Rev Microbiol 11: 209-250). It is known that fungal biosynthesis of fatty acids takes place in the cytosol and starts with carboxylation of acetyl-CoA to malonyl-CoA. From this malonyl-CoA consecutive C<sub>2</sub> units are added to acetyl-CoA or the growing fatty-CoA ester chain by a complex fatty  
20 acid synthase complex harboring seven different enzymatic activities. In contrast, knowledge of lipid biosynthesis and degradation has come from research in other organisms.

By way of example, it has been recognized that the biosynthesis of very long chain fatty acids in organisms  
25 other than fungi requires four enzyme systems: acetyl coenzyme A (CoA) carboxylase, fatty acid synthetase, fatty acid desaturase, and fatty acyl chain elongation system. The rate limiting step of the *de novo* synthesis of fatty acids is under the control of the first of these, acetyl-CoA  
30 carboxylase (EC 6.4.1.2). This enzyme catalyses the ATP-dependent carboxylation of acetyl CoA to yield malonyl CoA which serves as the two carbon unit donor for the subsequent synthesis of long chain fatty acids by the fatty acid

synthase complex. The chain length of newly synthesized fatty acids appears to depend on the concentration of malonyl-CoA rather than on the activity of the fatty acid synthase complex. Acetyl-CoA carboxylase thus regulates both  
5 the overall rate of de novo synthesis and chain length distribution of long chain fatty acids.

Acetyl-CoA carboxylase has been isolated from chicken liver (Buckner and Kolattakudy 1976 Biochem 15: 1948-1957; Manning et al 1976 Biochem J 153: 463-468; Ahmad et al 1978 J  
10 Biol Chem 253: 1733-1737; Hardie and Cohen 1978 FEBS Lett 91: 1-7); rat heart (Thampy 1989 J Biol Chem 264: 17631-17634); brown and white adipose tissue (Bianchi et al 1990 J Biol Chem 265: 1502-1509; Iverson et al 1990 Biochem J 269: 365-371); chick embryo brain (Thampy and Koshy 1991 J Lipid  
15 Res 32: 1667-1673) and has been observed by immunological techniques in rat diaphragm muscle (Bianchi et al 1990 *ibid*). Acetyl-CoA carboxylase has also been found in human skeletal muscle and adipose tissue (Witters et al 1994 Int J Biochem 26: 589-594) and in rat skeletal muscle (Trumble et al 1991  
20 Life Sci 49: 39-43).

Data have been accumulating from several laboratories characterizing the different isoforms of Acetyl-CoA carboxylase. Thampy (1989 *ibid*) and Bianchi et al (1990 *ibid*) have reported a molecular mass of 280 kDa for the  
25 Acetyl-CoA carboxylases from rat heart and diaphragm muscle respectively. More recently, two isoforms (HACC275 and HACC 265) have been identified in human tissue. The HACC 275 form is predominant in human skeletal muscle (Witters et al 1994 *ibid*). The rat skeletal muscle isoform appears to be similar  
30 in molecular mass to the HACC 275 form in humans. However, it has been recognized that until the Acetyl-CoA carboxylase gene(s) from each tissue are cloned and the mRNA species are characterized, assumption of equivalency of isoforms with

molecular masses is conjectural (Trumble et al 1995 Eur J Biochem 231: 192-198).

Preliminary studies on Acetyl-CoA carboxylase (Acc1p) from yeast *Saccharomyces* has been shown to: (i) have a  
5 subunit molecular mass of 250 kDa, (ii) be active as a tetramer and (iii) be subject to short term regulation by phosphorylation (Al-Feel et al 1992 Proc Natl Acad Sci 89: 4534-4538; Obernayer and Lynen 1976: Trends Biochem Sci 1: 169-171; Witters et al 1990 Biochem Biophys Res Commun 169:  
10 369-376). Genetic and biochemical analyses of fatty acid synthesis mutants and a conditional mRNA transport mutant of *Saccharomyces cerevisiae*, acc1-7-1, have also indicated that the continued synthesis of malonyl-CoA, the enzymatic product of acetyl-CoA carboxylase, is an essential function of the  
15 acetyl-CoA carboxylase (ACC1) gene (Schneiter et al 1996 Mol and Cell Biol 16: 7161-7172).

### Summary of the Invention

The invention is based on the discovery of an ACCase gene (afACC1) in the fungus *Aspergillus fumigatus*, which is essential for survival. Essential genes are genes which are  
5 required for growth (such as metabolism, division, or reproduction) and survival of an organism. Essential genes can be used to identify therapeutic antifungal agents. These therapeutic agents can reduce or prevent growth, or decrease pathogenicity or virulence, and preferably, kill the  
10 organism.

The *A. fumigatus* ACCase (afACCase) coding sequence is depicted in Fig. 1 as SEQ ID NO:1, with the amino acid sequence represented by SEQ ID NO:2. Thus the present invention relates to a novel ACCase enzyme - which is  
15 specific to *A. fumigatus* - and to a nucleotide sequence (afACC1) encoding same. The present invention also relates to the use of the novel nucleic acid and amino acid sequences in the diagnosis and treatment of disease. The present invention also relates to the use of the novel nucleic acid  
20 and amino acid sequences to evaluate and/or to screen for agents that can modulate ACCase activity. The present invention further relates to genetically engineered host cells that include or express the novel nucleic acid and amino acid sequences to evaluate and/or to screen for agents  
25 that can modulate ACCase activity.

The ACCase enzyme of the present invention is obtainable from the *A. fumigatus* fungal species. This ACCase enzyme is distinguishable from the Acetyl-CoA carboxylase enzymes identified in human skeletal and adipose tissue and the yeast  
30 (*S. cerevisiae*) Acetyl-CoA carboxylase known as Acc1p.

The ACCase enzyme of the present invention may be the same as the naturally occurring form - for this aspect, e.g., the ACCase can be the non-native amino acid sequence - or a

variant, homolog, fragment or derivative thereof. In addition, or in the alternative, the ACCase is isolated ACCase and/or purified ACCase. The ACCase can be obtainable from or produced by any suitable source, whether natural or  
5 not, or it may be synthetic, semi-synthetic or recombinant.

The ACCase gene of the invention is essential for survival of *A. fumigatus*. Accordingly, the ACCase nucleic acid sequence of the invention, and the ACCase polypeptide of the invention, are useful targets for identifying compounds  
10 that are inhibitors of *A. fumigatus*. Such inhibitors attenuate fungal growth by inhibiting the activity of the ACCase polypeptide, or by inhibiting transcription or translation. Accordingly, in one aspect, this invention provides isolated nucleic acid molecules encoding *A.*  
15 *fumigatus* ACCase polypeptides or biologically active portions thereof, as well as nucleic acid fragments suitable as primers or hybridization probes for the detection of ACCase-encoding nucleic acids (e.g., fragments of at least 15 nucleotides (e.g., at least 18, 20, or 25 nucleotides)).

20 The invention features a nucleic acid molecule that is at least 65% (or 75%, 85%, 95%, 98%, or 100%) identical to the nucleotide sequence shown in SEQ ID NO:1, or the nucleotide sequence of the cDNA insert of the plasmid deposited with the ATCC (10801 University Blvd., Manassas, VA  
25 209110-2209, USA) on December 15, 1998 as Accession Number 207005, 207006, 207007, 207008, or 207009 (the "cDNA of ATCC 207005, 207006, 207007, 207008, or 207009"), or a complement thereof. The deposited biological samples contain *E. coli* cells containing the plasmid EpAFACC-1, EpAFACC-2, EpAFACC-3,  
30 EpAFACC-4, and EpAFACC-5, respectively. Each EpAFACC plasmid contains a partial cDNA sequence of *A. fumigatus* ACCase, with the five plasmids together providing a complete cDNA sequence of *A. fumigatus* ACCase.

The invention features a nucleic acid molecule that includes a fragment of at least 300 (e.g., 325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1200, 1400, 1600, or 1770) nucleotides of the nucleotide sequence  
5 shown in SEQ ID NO:1, or the nucleotide sequence of the cDNA ATCC 207005, 207006, 207007, 207008, or 207009, or a complement thereof.

The invention also features a nucleic acid molecule that includes a nucleotide sequence encoding a protein having an  
10 amino acid sequence that is at least 65% (or 75%, 85%, 95%, 98%, or 100%) identical to the amino acid sequence of SEQ ID NO:2 or the amino acid sequence encoded by the cDNA of ATCC 207005, 207006, 207007, 207008, or 207009.

Also within the invention is a nucleic acid molecule  
15 that encodes a fragment of a polypeptide having the amino acid sequence of SEQ ID NO:2, the fragment including at least 15 (25, 30, 50, 100, 150, 300, 400, or 450) contiguous amino acids of SEQ ID NO:2 or the polypeptide encoded by the cDNA of ATCC Accession Number 207005, 207006, 207007, 207008, or  
20 207009.

In other embodiments, the invention features an isolated ACCase protein having an amino acid sequence that is at least about 65% (e.g., 75%, 85%, 95%, 98%, or 100%) identical to the amino acid sequence of SEQ ID NO:2; and an isolated  
25 ACCase protein which is encoded by a nucleic acid molecule having a nucleotide sequence that is at least about 65% (e.g., 75%, 85%, 95%, or 100%) identical to SEQ ID NO:1 or the cDNA of ATCC 207005, 207006, 207007, 207008, or 207009; and an isolated ACCase protein which is encoded by a nucleic  
30 acid molecule having a nucleotide sequence which hybridizes under stringent hybridization conditions to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1 or the

non-coding strand of the cDNA of ATCC 207005, 207006, 207007, 207008, or 207009.

Another embodiment of the invention features ACCase nucleic acid molecules that specifically detect *A. fumigatus* 5 ACCase nucleic acid molecules relative to nucleic acid molecules encoding other ACCases. For example, in one embodiment, an *A. fumigatus* ACCase nucleic acid molecule hybridizes under stringent conditions to a nucleic acid molecule that includes the nucleotide sequence of SEQ ID 10 NO:1, or the cDNA of ATCC 207005, 207006, 207007, 207008, or 207009, or a complement thereof. In another embodiment, the *A. fumigatus* ACCase nucleic acid molecule is at least 300 (e.g., 400, 500, 700, 900, 1100, or 1300) nucleotides in length and hybridizes under stringent conditions to a nucleic 15 acid molecule that includes the nucleotide sequence shown in SEQ ID NO:1, the cDNA of ATCC 207005, 207006, 207007, 207008, or 207009, or a complement thereof. In another embodiment, the invention provides an isolated nucleic acid molecule which is antisense to the coding strand of an *A. fumigatus* 20 ACCase nucleic acid.

Another aspect of the invention provides a vector, e.g., a recombinant expression vector, that includes an ACCase nucleic acid molecule of the invention. In another embodiment the invention provides a host cell containing such 25 a vector. The invention also provides a method for producing ACCase protein by culturing, in a suitable medium, a host cell of the invention containing a recombinant expression vector such that an ACCase protein is produced.

Another aspect of this invention features isolated or 30 recombinant ACCase proteins and polypeptides. Typical ACCase proteins and polypeptides possess at least one biological activity possessed by naturally occurring *A. fumigatus* ACCase, e.g., an ability to synthesize malonyl CoA from

acetyl CoA. It is not necessary that the ACCase polypeptide have activity that is equivalent to that of the wild-type *A. fumigatus* ACCase. For example, the ACCase polypeptide can have 20, 50, 75, 90, 100, or an even higher percent of the wild-type activity.

Since the *A. fumigatus* ACCase gene, which is essential for survival, has been identified, nucleic acids encoding *A. fumigatus* ACCase and *A. fumigatus* ACCase proteins can be used to identify antifungal agents. Such antifungal agents can be identified with high throughput assays to detect inhibition of ACCase activity. For example, this inhibition can be caused by small molecules binding directly to the ACCase polypeptide or by binding of small molecules to other essential polypeptides in a biochemical pathway in which ACCase participates.

The invention also provides methods of identifying agents (such as compounds, other substances, or compositions) that affect, or selectively affect, (such as inhibit or otherwise modify) the activity of and/or expression of the ACCase, by contacting the ACCase or the nucleotide sequence coding for same with the agent and then measuring the activity of the ACCase and/or the expression thereof. In a related aspect, the invention features a method of identifying agents (such as compounds, other substances or compositions comprising same) that affect (such as inhibit or otherwise modify) the activity of and/or expression of afACCase, by measuring the activity of and/or expression of afACCase in the presence of the agent or after the addition of the agent in: (a) a cell line into which has been incorporated a recombinant construct including the nucleotide sequence of the afACCase gene (e.g., SEQ ID NO:1) or an allelic variation thereof, or (b) a cell population or cell line that naturally selectively expresses afACCase, and then



measuring the activity of afACCCase and/or the expression thereof.

Since the *Aspergillus fumigatus* ACCCase gene has been identified, it can be cloned into various host cells (e.g.,  
5 fungi, *E. coli* or yeast) for carrying out such assays in whole cells). Similarly, conventional in vitro assays of ACCCase activity can be used with the ACCCase of the invention.

In one embodiment, the invention features a method for identifying a compound for the treatment of a fungal  
10 infection, wherein the method entails, in sequence, (i) preparing a first cell and a second cell, the first and second cells being capable of expressing afACCCase, (ii) contacting the first cell with a test compound, (iii) determining the level of expression of afACCCase in the first  
15 and second cells, (iv) comparing the level of expression in the first cell with the second cell, and (v) selecting the test compound for treatment of a fungal infection where expression of afACCCase in the first cell is less than expression of the essential gene in the second cell, and  
20 wherein the afACCCase gene is a first nucleic acid molecule which encodes a polypeptide including the amino acid sequence of SEQ ID NO: 2, or a naturally occurring allelic variant thereof, and wherein the first nucleic acid molecule hybridizes under stringent conditions to a second nucleic  
25 acid molecule, the second nucleic acid molecule consisting of a nucleotide sequence of SEQ ID NO: 1. The determination of the level of expression of the afACCCase gene can be made by measuring the amount of mRNA transcribed from the afACCCase gene. Alternatively, the level of afACCCase encoded by the  
30 afACCCase gene can be measured.

The test compound can be a small organic molecule. Alternatively, the test compound can be a test polypeptide (e.g., a polypeptide having a random or predetermined amino

acid sequence; or a naturally-occurring or synthetic polypeptide) or a nucleic acid, such as a DNA or RNA molecule. The test compound can be a naturally-occurring compound or it can be synthetically produced. Synthetic  
5 libraries, chemical libraries, and the like can be screened to identify compounds that bind to ACCase.

In another suitable method, there is provided an assay method for identifying an agent that can affect Acetyl CoA Carboxylase (ACC) activity or expression thereof, the assay  
10 method comprising contacting an agent with an amino acid sequence according to the present invention or a nucleotide sequence according to the present invention; and measuring the activity or expression of ACC; wherein a difference in activity between a) ACCase activity or expression in the  
15 absence of the agent and b) ACCase activity or expression in the presence of the agent is indicative that the agent can affect ACCase activity or expression.

Another suitable method for identifying antifungal compounds involves screening for small molecules that  
20 specifically bind to ACCase. A variety of suitable binding assays are known in the art as described, for example, in U.S. Patent Nos. 5,585,277 and 5,679,582, hereby incorporated herein by reference. For example, in various conventional assays, test compounds can be assayed for their ability to  
25 bind a polypeptide by measuring the ability of the small molecule to stabilize the polypeptide in its folded, rather than unfolded, state. More specifically, one can measure the degree of protection against unfolding that is afforded by the test compound. Test compounds that bind afACCase with  
30 high affinity cause, for example, a significant shift in the temperature at which the polypeptide is denatured. Test compounds that stabilize the polypeptide in a folded state

can be further tested for antifungal activity in a standard susceptibility assay.

In a related method for identifying antifungal compounds, an ACCase polypeptide is used to isolate peptide  
5 or nucleic acid ligands that specifically bind to the ACCase polypeptides. These peptide or nucleic acid ligands are then used in a displacement screen to identify small molecules that bind to the ACCase polypeptide. Such binding assays can be carried out as described herein.

10 The *A. fumigatus* ACCase polypeptides also can be used in assays to identify test compounds that bind to the polypeptides. Test compounds that bind to the ACCase polypeptides then can be tested, in conventional assays, for their ability to inhibit fungal growth. Test compounds that  
15 bind to the ACCase polypeptides are candidate antifungal agents, in contrast to compounds that do not bind to the ACCase polypeptides. As described herein, any of a variety of art-known methods can be used to assay for binding of test compounds to the ACCase polypeptides.

20 The invention includes, for example, a method for identifying a compound useful for treating a fungal infection, wherein the method entails (a) measuring the level of expression of the afACCase gene in a cell in the presence of a test compound; (b) comparing the level of expression  
25 measured in step (a) to the level of expression of the afACCase gene in a cell in the absence of the test compound; and (c) selecting the test compound as being useful for treating a fungal infection when the level of expression of the afACCase gene in the presence of the test compound is  
30 less than the level expression of the afACCase gene in the absence of the test compound, and wherein the afACCase gene has the sequence of SEQ ID NO: 1. If desired, the level of expression can be measured by measuring the amount of mRNA

from the afACCCase gene described herein, or by measuring the amount of protein encoded by the afACCCase gene described herein. Typically, the cell is *A. fumigatus* or *Saccharomyces* (e.g., *Saccharomyces cerevisiae*).

5 In a variation of the above method, the invention features a method for identifying a compound useful for treating a fungal infection, wherein the method entails (a) measuring the activity of the afACCCase gene in a cell in the presence of a test compound; (b) comparing the activity  
10 measured in step (a) to the level activity of the afACCCase gene in a cell in the absence of the test compound; and (c) selecting the test compound as being useful for treating fungal infections when the level of activity of the afACCCase gene measured in the presence of the test compound is less  
15 than the level of activity of the afACCCase gene measured in the absence of the test compound, wherein the afACCCase gene has the sequence of SEQ ID NO: 1.

In an alternative method, the invention features a method for identifying a compound useful for treating a  
20 fungal infection, wherein the method entails (a) measuring, in the presence of a test compound, the growth of a sample of cells which have been engineered to express a afACCCase gene; (b) comparing the growth measured in step (a) to the growth of a sample of the cells in the absence of the test compound;  
25 and (c) selecting the test compound as being useful for treating a fungal infection when the growth of the sample of cells in the presence of the test compound is slower than the growth of a sample of cells in the absence of the test compound, wherein the afACCCase gene has the sequence of SEQ  
30 ID NO: 1. Typically, the cell sample contains fungal cells (e.g., *A. fumigatus*).

The invention also includes a method for identifying an antifungal agent where the method entails: (a) contacting an

ACCCase polypeptide with a test compound; (b) detecting binding of the test compound to the polypeptide; and (c) determining whether a test compound that binds to the polypeptide inhibits growth of *A. fumigatus*, relative to growth of fungi cultured in the absence of the test compound, as an indication that the test compound is an antifungal agent. If desired, the test compound can be immobilized on a substrate, and binding of the test compound to afACCase is detected as immobilization of afACCase on the immobilized test compound. Immobilization of afACCase on the test compound can be detected in an immunoassay with an antibody that specifically binds to afACCase.

In still another method, binding of a test compound to an ACCase polypeptide can be detected in a conventional two-hybrid system for detecting protein/protein interactions (e.g., in yeast or mammalian cells). A test compound found to bind to afACCase can be further tested for antifungal activity in a conventional susceptibility assay. Generally, in such two-hybrid methods, (a) afACCase is provided as a fusion protein that includes the polypeptide fused to (i) a transcription activation domain of a transcription factor or (ii) a DNA-binding domain of a transcription factor; (b) the test polypeptide is provided as a fusion protein that includes the test polypeptide fused to (i) a transcription activation domain of a transcription factor or (ii) a DNA-binding domain of a transcription factor; and (c) binding of the test polypeptide to the polypeptide is detected as reconstitution of a transcription factor. Reconstitution of the transcription factor can be detected, for example, by detecting transcription of a gene that is operably linked to a DNA sequence bound by the DNA-binding domain of the reconstituted transcription factor (See, for example, White, 1996, Proc. Natl. Acad. Sci. 93:10001-10003 and references

cited therein and Vidal et al., 1996, Proc. Natl. Acad. Sci. 93:10315-10320).

In an alternative method, an isolated nucleic acid molecule encoding an ACCase is used to identify a compound  
5 that decreases the expression of ACCase *in vivo* (i.e., in an *A. fumigatus* cell). Such compounds can be used as antifungal agents. To discover such compounds, cells that express an ACCase are cultured, exposed to a test compound (or a mixture of test compounds), and the level of ACCase expression or  
10 activity is compared with the level of ACCase expression or activity in cells that are otherwise identical but that have not been exposed to the test compound(s). Standard quantitative assays of gene expression and ACCase activity can be utilized in this aspect of the invention.

15 To identify compounds that modulate expression of ACCase the test compound(s) can be added at varying concentrations to the culture medium of *A. fumigatus*. Such test compounds can include small molecules (typically, non-protein, non-polysaccharide chemical entities), polypeptides, and nucleic  
20 acids. The expression of ACCase is then measured, for example, by Northern blot PCR analysis or RNase protection analyses using a nucleic acid molecule of the invention as a probe. The level of expression in the presence of the test molecule, compared with the level of expression in its  
25 absence, will indicate whether or not the test molecule alters the expression of ACCase. Because ACCase is essential for survival, test compounds that inhibit the expression and/or function of ACCase will inhibit growth of, or kill, the cells that express ACCase.

30 More generally, binding of a test compound to an ACCase polypeptide can be detected either *in vitro* or *in vivo*. If desired, the above-described methods for identifying compounds that modulate the expression of the ACCase

polypeptides of the invention can be combined with measuring the levels of ACCase expressed in cells, e.g., by carrying out an assay of ACCase activity, as described above or, for example, performing a Western blot analysis using antibodies  
5 that bind to ACCase. The antifungal agents identified by the methods of the invention can be used to inhibit a wide spectrum of pathogenic or non-pathogenic fungal strains.

The invention also features a method for identifying an antifungal agent, where the method entails (a) contacting an  
10 afACCase polypeptide with a test compound; (b) detecting a decrease in activity of afACCase the contacted with test compound; (c) selecting a compound useful for treating a fungal infection as one that decreases the activity of afACCase; and, optionally, (d) determining whether a test  
15 compound that decreases activity of a contacted afACCase polypeptide inhibits growth of fungi, relative to growth of fungi cultured in the absence of a test compound that decreases activity of a contacted ACCase polypeptide, wherein inhibition of growth indicates that the test compound is an  
20 antifungal agent, and wherein afACCase is encoded by a gene having the sequence of SEQ ID NO: 1. The test compound can be, without limitation, a polypeptide, ribonucleic acid, small molecule, deoxyribonucleic acid, antisense oligonucleotide, or ribozyme.

25 In yet another embodiment, the invention features a method for identifying a compound useful for treating a fungal infection, wherein the method entails (a) contacting a variant, homolog, or ortholog of an ACCase polypeptide with a test compound; (b) detecting binding of the test compound  
30 to the variant, homolog, or ortholog of afACCase; and (c) selecting a compound useful for treating a fungal infection as one that binds to the variant, homolog, or ortholog of afACCase, wherein afACCase is encoded by a gene having the

sequence of SEQ ID NO: 1. Optionally, the method can also include (d) determining whether a test compound that binds to the variant, homolog, or ortholog of afACCCase inhibits growth of fungi, relative to growth of fungi cultured in the absence of a test compound that binds to the variant, homolog, or ortholog of afACCCase, wherein inhibition of growth indicates that the test compound is an antifungal agent. The variant, homolog, or ortholog can be derived from a non-pathogenic, or pathogenic, fungus.

Some specific embodiments of the present invention relate to assay methods for the identification of antifungal agents using assays for antifungal agents which may be carried out both in whole cell preparations and in ex vivo cell-free systems. In each instance, the assay target is the ACCase nucleotide sequence - which is essential for fungal viability - and/or the ACCase polypeptide. Candidate agents which are found to inhibit the target nucleotide sequence and/or afACCCase in any assay method of the present invention are thus identified as potential antifungal agents. It is expected that the assay methods of the present invention will be suitable for both small and large-scale screening of test compounds as well as in quantitative assays such as serial dilution studies wherein the target ACCase nucleotide sequence or the ACCase polypeptide are exposed to a range of candidate agent concentrations.

When the assay methods of the present invention are carried out as a whole-cell assay, the target ACCase nucleotide sequence and/or the ACCase polypeptide and the entire living fungal cell may be exposed to the candidate agent under conditions normally suitable for growth. Optimal conditions including essential nutrients, optimal temperatures and other parameters, depend upon the particular fungal strain being used and suitable conditions are well



known in the art. Inhibition of expression of the target nucleotide sequence and/or the activity of afACCCase may be determined in a number of ways including observing the cell culture's growth or lack thereof. Such observation may be  
5 made visually, by optical densitometric or other light absorption/scattering means or by yet other suitable means, whether manual or automated.

In the above whole-cell assay, an observed lack of cell growth may be due to inhibition of the target nucleotide  
10 sequence and/or afACCCase or may be due to an entirely different effect of the candidate agent, and further evaluation may be required to establish the mechanism of action and to determine whether the candidate agent is a specific inhibitor of the target. Accordingly, and in one  
15 embodiment of the present invention, the method may be performed as a paired-cell assay in which each test compound is separately tested against two different fungal cells, the first fungal cells having a target with altered properties making it more susceptible to inhibition compared with that  
20 of the second fungal cells.

One manner of achieving differential susceptibility is by using mutant strains expressing a modified target ACCase polypeptide. A particularly useful strain is one having a temperature sensitive ("ts") mutation as a result of which  
25 the target is more prone than the wild type target to loss of functionality at high temperatures (that is, temperatures higher than optimal, but still permitting growth in wild type cells). When grown at semi-permissive temperatures, the activity of a ts mutant target may be attenuated but  
30 sufficient for growth.

Alternatively or in conjunction with the above, differential susceptibility to target inhibitors may be obtained by using a second fungal cell which has altered

properties making it less susceptible to inhibition compared with that of wild type cells as for example, a fungal cell which has been genetically manipulated to cause overexpression of the target. Such overexpression can be  
5 achieved by placing into a wild type cell a plasmid carrying the nucleotide sequence for the target. The techniques for generating temperature sensitive mutants, for preparing specific plasmids and for transforming cell lines with such plasmids are well known in the art.

10 Alternatively or in conjunction with the above, the access of candidate agents to a cell or an organism, may be enhanced by mutating or deleting a gene or genes which encode a protein or proteins responsible for providing a permeability barrier for a cell or an organism.

15 The present invention also relates to a method for identifying antifungal agents utilizing fungal cell systems that are sensitive to perturbation to one or several transcriptional/translational components.

By way of example, the present invention relates to a  
20 method of constructing mutant fungal cells in which one or more of the transcriptional/translational components is present in an altered form or in a different amount compared with a corresponding wild-type cell. This method further involves examining a candidate agent for its ability to  
25 perturb transcription/translation by assessing the impact it has on the growth of the mutant and wild-type cells. Agents that perturb transcription/translation by acting on a particular component that participates in transcription/translation may cause a mutant fungal cell  
30 which has an altered form or amount of that component to grow differently from the corresponding wild-type cell, but do not affect the growth relative to the wild type cell of other mutant cells bearing alterations in other components

participating in transcription/translation. This method thus provides not only a means to identify whether a candidate agent perturbs transcription/translation but also an indication of the site at which it exerts its effects. The  
5 transcriptional/translational component which is present in altered form or amount in a cell whose growth is affected by a candidate agent is likely to be the site of action of the agent.

By way of example, the present invention provides a  
10 method for identifying antifungal agents which interfere with steps in translational accuracy, such as maintaining a proper reading frame during translation and terminating translation at a stop codon. This method involves constructing mutant fungal cells in which a detectable reporter polypeptide can  
15 only be produced if the normal process of staying in one reading frame or of terminating translation at a stop codon has been disrupted. This method further involves contacting the mutant fungal cells with a candidate agent to examine whether it increases or decreases the production of the  
20 reporter polypeptide.

The present invention also provides a method of screening an agent for specific binding affinity with afACCCase (or a derivative, homolog, variant or fragment thereof) or the nucleotide sequence coding for same  
25 (including a derivative, homolog, variant or fragment thereof), the method comprising the steps of: a) providing a candidate agent; b) combining afACCCase (or the derivative, homolog, variant or fragment thereof) or the nucleotide sequence coding for same (or the derivative, homolog, variant  
30 or fragment thereof) with the candidate agent for a time sufficient to allow binding under suitable conditions; such binding or interaction being associated with a second component capable of providing a detectable signal in

response to the binding or interaction of the ACCase polypeptide or the nucleotide sequence encoding same with the agent; and c) determining whether the agent binds to or otherwise interacts with and activates or inhibits an activity of afACCase (or the derivative, homolog, variant or fragment thereof) or the expression of the nucleotide sequence coding for same (or the derivative, homolog, variant or fragment thereof) by detecting the presence or absence of a signal generated from the binding and/or interaction of the agent with afACCase (or the derivative, homolog, variant or fragment thereof) or the nucleotide sequence coding for same (or the derivative, homolog, variant or fragment thereof).

In other embodiments, the cell system is an extract of a fungal cell that is grown under defined conditions, and the method involves measuring transcription or translation in vitro. Such defined conditions are selected so that transcription or translation of the reporter is increased or decreased by the addition of a transcription inhibitor or a translation inhibitor to the cell extract.

One such method for identifying antifungal agents relies upon a transcription-responsive gene product. This method involves constructing a fungal cell in which the production of a reporter molecule, measured as a percentage of over-all transcription, increases or decreases under conditions in which overall fungal cell transcription is reduced. Specifically, the reporter molecule is encoded by a nucleic acid transcriptionally linked to a sequence constructed and arranged to cause a relative increase or decrease in the production of the reporter molecule when overall transcription is reduced. Typically, the overall transcription is measured by the expression of a second indicator gene whose expression, when measured as a percentage of overall transcription, remains constant when

the overall transcription is reduced. The method further involves contacting the fungal cell with a candidate agent, and determining whether the agent increases or decreases the production of the first reporter molecule in the fungal cell.

5 In one embodiment, the reporter molecule is itself the transcription-responsive gene product whose production increases or decreases when overall transcription is reduced. In another embodiment, the reporter is a different molecule whose production is linked to that of the transcription-  
10 responsive gene product. Such linkage between the reporter and the transcription-responsive gene product can be achieved in several ways. A gene sequence encoding the reporter may, for example, be fused to part or all of the gene encoding the transcription-responsive gene product and/or to part or all  
15 of the genetic elements which control the production of the gene product. Alternatively, the transcription-responsive gene product may stimulate transcription of the gene encoding the reporter, either directly or indirectly.

Alternatively, the method for identifying antifungal  
20 agents relies upon a translation-responsive gene product. This method involves constructing a fungal cell in which the production of a reporter molecule, measured as a percentage of over-all translation, increases or decreases under conditions in which overall fungal cell translation is  
25 reduced. Specifically, the reporter molecule is encoded by nucleic acid either translationally linked or transcriptionally linked to a sequence constructed and arranged to cause a relative increase or decrease in the production of the reporter molecule when overall translation  
30 is reduced. Typically, the overall translation is measured by the expression of a second indicator gene whose expression, when measured as a percentage of overall translation, remains constant when the overall translation is

reduced. The method further involves contacting the fungal cell with a candidate agent, and determining whether the agent increases or decreases the production of the first reporter molecule in the fungal cell.

5 In one embodiment, the reporter molecule is itself the translation-responsive gene product whose production increases or decreases when overall translation is reduced. In another embodiment, the reporter is a different molecule whose production is linked to that of the translation-  
10 responsive gene product. Such linkage between the reporter and the translation-responsive gene product can be achieved in several ways. A gene sequence encoding the reporter may, for example, be fused to part or all of the gene encoding the translation-responsive gene product and/or to part or all of  
15 the genetic elements which control the production of the gene product. Alternatively, the translation-responsive gene product may stimulate translation of the gene encoding the reporter, either directly or indirectly.

Generally, a wide variety of reporters may be used, with  
20 typical reporters providing conveniently detectable signals (eg. by spectroscopy). By way of example, a reporter gene may encode an enzyme which catalyses a reaction which alters light absorption properties.

Examples of reporter molecules include but are not  
25 limited to -galactosidase, invertase, green fluorescent protein, luciferase, chloramphenicol, acetyltransferase, beta-glucuronidase, exo-glucanase and glucoamylase. Alternatively, radiolabeled or fluorescent tag-labeled nucleotides can be incorporated into nascent transcripts  
30 which are then identified when bound to oligonucleotide probes. For example, the production of the reporter molecule can be measured by the enzymatic activity of the reporter gene product, such as -galactosidase.

In another embodiment of the present invention, a selection of hybridization probes corresponding to a predetermined population of genes of the selected fungal organism may be used to specifically detect changes in gene transcription which result from exposing the selected organism or cells thereof to a candidate agent. In this embodiment, one or more cells derived from the organism is exposed to the candidate agent *in vivo* or *ex vivo* under conditions wherein the agent effects a change in gene transcription in the cell to maintain homeostasis. Thereafter, the gene transcripts, primarily mRNA, of the cell or cells are isolated by conventional means. The isolated transcripts or cDNAs complementary thereto are then contacted with an ordered matrix of hybridization probes, each probe being specific for a different one of the transcripts, under conditions wherein each of the transcripts hybridizes with a corresponding one of the probes to form hybridization pairs. The ordered matrix of probes provides, in aggregate, complements for an ensemble of genes of the organism sufficient to model the transcriptional responsiveness of the organism to a candidate agent. The probes are generally immobilized and arrayed onto a solid substrate such as a microtiter plate. Specific hybridization may be effected, for example, by washing the hybridized matrix with excess non-specific oligonucleotides. A hybridization signal is then detected at each hybridization pair to obtain a transcription signal profile. A wide variety of hybridization signals may be used. In one embodiment, the cells are pre-labeled with radionucleotides such that the gene transcripts provide a radioactive signal that can be detected in the hybridization pairs. The transcription signal profile of the agent-treated cells is then compared with a transcription signal profile of negative control cells

to obtain a specific transcription response profile to the candidate agent.

A variety of protocols for detecting and measuring the expression of afACCCase, using either polyclonal or monoclonal  
5 antibodies specific for the protein, are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing  
10 monoclonal antibodies reactive to two non-interfering epitopes on afACCCase polypeptides is suitable; alternatively, a competitive binding assay may be employed. These and other assays are described, among other places, in Hampton R et al. (1990, Serological Methods, A Laboratory Manual, APS Press, St Paul MN) and Maddox DE et al. (1983, J. Exp. Med. 15  
15 8:121).

In an embodiment of the present invention, afACCCase or a variant, homolog, fragment or derivative thereof and/or a cell line that expresses afACCCase or variant, homolog, fragment or derivative thereof may be used to screen for  
20 antibodies, peptides, or other agents, such as organic or inorganic molecules, that act as modulators of afACCCase activity, thereby identifying a therapeutic agent capable of modulating the activity of afACCCase. For example, antibodies that specifically bind an ACCCase polypeptide and are capable  
25 of neutralizing the activity of afACCCase may be used to inhibit afACCCase activity. Alternatively, screening of peptide libraries or organic libraries made by combinatorial chemistry with recombinantly expressed ACCCase polypeptide or a variant, homolog, fragment or derivative thereof or cell  
30 lines expressing afACCCase or a variant, homolog, fragment or derivative thereof may be useful for identification of therapeutic agents that function by modulating afACCCase activity. Synthetic compounds, natural products, and other



sources of potentially biologically active materials can be screened in a number of ways deemed to be routine to those of skill in the art. For example, nucleotide sequences encoding the N-terminal region of afACCCase' can be expressed in a cell  
5 line and used for screening of allosteric modulators, either agonists or antagonists, of afACCCase activity.

Accordingly, the present invention provides a method for screening a plurality of agents for specific binding affinity with afACCCase, or a portion, variant, homolog, fragment or  
10 derivative thereof, by providing a plurality of agents; combining afACCCase or a portion, variant, homolog, fragment or derivative thereof with each of a plurality of agents for a time sufficient to allow binding under suitable conditions; and detecting binding of afACCCase, or portion, variant,  
15 homolog, fragment or derivative thereof, to each of the plurality of agents, thereby identifying the agent or agents which specifically bind afACCCase. In such an assay, the plurality of agents may be produced by combinatorial chemistry techniques known to those of skill in the art.

20 Another technique for screening provides for high throughput screening of agents having suitable binding affinity to afACCCase polypeptides and is based upon the method described in detail in WO 84/03564. In summary, large numbers of different small peptide test compounds are  
25 synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test agents are reacted with afACCCase fragments and washed. A bound ACCase polypeptide is then detected - such as by appropriately adapting methods well known in the art. A purified ACCase polypeptide can  
30 also be coated directly onto plates for use in the aforementioned drug screening techniques. Alternatively, non-neutralizing antibodies can be used to capture the peptide and immobilize it on a solid support.

Typically, in an antifungal discovery process, potential new antifungal agents are tested for their ability to inhibit the *in vitro* activity of the purified expression product of the present invention in a biochemical assay. Agents with  
5 inhibitory activity can then progress to an *in vitro* antifungal activity screening using a standard MIC (Minimum Inhibitory Concentration) test (based on the M27-A NCCLS approved method). Antifungal active agents identified at this point are then tested for antifungal efficacy *in vivo*,  
10 such as by using rodent systemic candidiasis/aspergillosis models. Efficacy is measured by measuring the agent's ability to increase the host animal's survival rate against systemic infection, and/or reduce the fungal burden in infected tissues, compared to control animals receiving no  
15 administered agent (which can be by oral or intravenous routes).

The present invention also provides a pharmaceutical composition for treating an individual in need of such treatment of a disease caused by afACCase activity (or that  
20 can be treated by inhibiting afACCase activity); the treatment method entails administering a therapeutically effective amount of an agent that affects (such as inhibits) the activity and a pharmaceutically acceptable carrier, diluent, excipient or adjuvant.

25 The pharmaceutical compositions can be used for humans or animals and will typically include any one or more of a pharmaceutically acceptable diluent, carrier, excipient or adjuvant. The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of  
30 administration and standard pharmaceutical practice. The pharmaceutical compositions can include as (or in addition to) the carrier, excipient, or diluent any suitable

binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilizing agent(s).

The invention includes pharmaceutical formulations that include a pharmaceutically acceptable excipient and an  
5 antifungal agent identified using the methods described herein. In particular, the invention includes pharmaceutical formulations that contain antifungal agents that inhibit the growth of, or kill, pathogenic fungal strains (e.g., pathogenic *Aspergillus fumigatus* strains). Such  
10 pharmaceutical formulations can be used in a method of treating a fungal infection in an organism. Such a method entails administering to the organism a therapeutically effective amount of the pharmaceutical formulation, i.e., an amount sufficient to ameliorate signs and/or symptoms of the  
15 fungal infection. In particular, such pharmaceutical formulations can be used to treat fungal infections in mammals such as humans and domesticated mammals (e.g., cows, pigs, dogs, and cats), and in plants. The efficacy of such antifungal agents in humans can be estimated in an animal  
20 model system well known to those of skill in the art (e.g., mouse systems of fungal infections).

The invention also includes (i) a method of treating a mycosal and/or fungal infection in a target (which target can be a living organism, such as a mammal, such as a human, or  
25 an inanimate target, such as a textile piece, paper, plastic etc.), which method entails delivering (such as administering or exposing) an effective amount of an agent capable of modulating the expression pattern of the nucleotide sequence of the present invention or the activity of the expression  
30 product thereof; and (ii) a method of treating a mycosal and/or fungal infection in a target (which target can be a living organism, such as a plant or a mammal, such as a human, or an inanimate target, such as a textile piece,

paper, plastic etc.), which method entails delivering (such as administering or exposing) an effective amount of an agent identified by an assay according to the present invention. As used herein, the terms "treating," "treat," or "treatment" 5 include, inter alia, preventative (e.g., prophylactic), palliative, and curative treatment of fungal infections.

The invention also features a method for inducing an immunological response in an individual, particularly a mammal, which entails inoculating the individual with one or 10 more of the ACCase genes or polypeptides described herein, and generally in an amount adequate to produce an antibody and/or T cell immune response to protect the individual from mycoses, fungal infection, or infestations. In another aspect, the present invention relates to a method of inducing 15 an immunological response in an individual which entails delivering to the individual a vector that includes an ACCase gene described herein or a variant, homolog, fragment, or derivative thereof in vivo to induce an immunological response, such as to produce antibody and/or a T-cell immune 20 response to protect the individual from disease whether that disease is already established within the individual or not.

Various affinity reagents that are permeable to the microbial membrane (i.e., antibodies and antibody fragments) are useful in practicing the methods of the invention. For 25 example polyclonal and monoclonal antibodies that specifically bind to the *A. fumigatus* ACCase polypeptide can facilitate detection of *A. fumigatus* ACCase in various fungal strains (or extracts thereof). These antibodies also are useful for detecting binding of a test compound to ACCase 30 (e.g., using the assays described herein). In addition, monoclonal antibodies that specifically bind to *A. fumigatus* ACCase can themselves be used as antifungal agents.

In another aspect, the invention features a method for detecting an *A. fumigatus* ACCase polypeptide in a sample. This method includes: obtaining a sample suspected of containing an *A. fumigatus* ACCase polypeptide; contacting the  
5 sample with an antibody that specifically binds to an *A. fumigatus* ACCase polypeptide under conditions that allow the formation of complexes of the antibody and the ACCase polypeptide; and detecting the complexes, if any, as an indication of the presence of an *A. fumigatus* ACCase  
10 polypeptide in the sample.

In all of the foregoing methods, homologs, orthologs, or variants of the ACCase genes and polypeptides described herein can be substituted. While "homologs" are structurally similar genes contained within a species, "orthologs" are  
15 functionally equivalent genes from other species (within or outside of a given genus, e.g., from *E. coli*). The terms "variant," "homolog," or "fragment" in relation to the amino acid sequence of the ACCase of the invention include any substitution, variation, modification, replacement, deletion,  
20 or addition of one or more amino acids from or to the sequence providing the resultant ACCase polypeptide.

The invention offers several advantages. The invention provides targets, based on essential functions, for identifying potential agents for the effective treatment of  
25 opportunistic infections caused by *A. fumigatus* and other related fungal species. Also, the methods for identifying antifungal agents can be configured for high throughput screening of numerous candidate antifungal agents. Because the ACCase gene disclosed herein is thought to be highly  
30 conserved, antifungal drugs targeted to this gene or its gene products are expected to have a broad spectrum of antifungal activity.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or  
5 equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated herein by  
10 reference in their entirety. In the case of a conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative and are not intended to limit the scope of the invention, which is defined by the claims.  
15 Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

#### Brief Description of the Drawing

20 Fig. 1 is a listing of the nucleotide sequence (SEQ ID NO:1) and predicted amino acid sequence (SEQ ID NO:2) of *Aspergillus fumigatus* (afACCCase).

#### Detailed Description of the Invention

25 A gene encoding acetyl coenzyme A carboxylase of *Aspergillus fumigatus* has been identified and is essential for the survival of *A. fumigatus*. The ACCase gene and polypeptide are useful targets for identifying compounds that are inhibitors of the fungi in which ACCase polypeptides are  
30 expressed.

Nucleic acids described herein include both RNA and DNA, including genomic DNA and synthetic (e.g., chemically synthesized) DNA. Nucleic acids can be double-stranded or

single-stranded. Where single-stranded, the nucleic acid can be a sense strand or an antisense strand. Nucleic acids can be synthesized using oligonucleotide analogs or derivatives (e.g., inosine or phosphorothioate nucleotides). Such  
5 oligonucleotides can be used, for example, to prepare nucleic acids that have altered base-pairing abilities or increased resistance to nucleases.

An isolated nucleic acid is a DNA or RNA that is not immediately contiguous with both of the coding sequences with  
10 which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. Thus, in one embodiment, an isolated nucleic acid includes some or all of the 5' non-coding (e.g., promoter) sequences that are immediately  
15 contiguous to the coding sequence. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a genomic DNA  
20 fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA that is part of a hybrid gene encoding an additional polypeptide sequence. The terms "isolated" and "purified" refer to a nucleic acid or polypeptide that is  
25 substantially free of cellular or viral material with which it is naturally associated, or culture medium (when produced by recombinant DNA techniques), or chemical precursors or other chemicals (when chemically synthesized). Moreover, an isolated nucleic acid fragment is a nucleic acid fragment  
30 that is not naturally occurring as a fragment and would not be found in the natural state.

A nucleic acid sequence that is substantially identical to an ACCase nucleotide sequence is at least 80% identical to

the nucleotide sequence of ACCase as represented by the SEQ ID NO:1, as depicted in Fig. 1. For purposes of comparison of nucleic acids, the length of the reference nucleic acid sequence will generally be at least 40 nucleotides, e.g., at  
5 least 60 nucleotides or more nucleotides.

To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid  
10 sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the  
15 corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = # of identical positions/total # of overlapping  
20 positions x 100). Preferably, the two sequences are the same length.

The determination of percent identity or homology between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of  
25 a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) *Proc. Nat'l Acad. Sci. USA* 87:2264-2268, modified as in Karlin and Altschul (1993) *Proc. Nat'l Acad. Sci. USA* 90:5873-5877. Such an algorithm is incorporated into the  
30 NBLAST and XBLAST programs of Altschul, et al. (1990) *J. Mol. Biol.* 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to ACCase



nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to ACCase protein molecules of the invention. To obtain  
5 gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) *Nucleic Acids Res.* 25:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See  
10 <http://www.ncbi.nlm.nih.gov>. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence  
15 alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

The percent identity between two sequences can be  
20 determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, only exact matches are counted.

The terms "variant," "homolog," or "fragment" in relation to the nucleotide sequence encoding an ACCase of the  
25 present invention include any substitution, variation, modification, replacement, deletion, or addition of one (or more) nucleotides from or to the sequence of an ACCase gene. Typically, the resultant nucleotide sequence encodes or is capable of encoding an ACCase polypeptide that generally is  
30 at least as biologically active as the referenced ACCase polypeptide (e.g., as represented by SEQ ID NO:2). In particular, the term "homolog" covers homology with respect to structure and/or function providing the resultant

nucleotide sequence codes for or is capable of coding for an ACCase polypeptide being at least as biologically active as afACCase encoded by the sequence shown as SEQ ID NO:1. With respect to sequence homology, there is at least 75% (e.g.,  
5 85%, 90%, 95%, 98%, or 100%) homology to the sequence shown as SEQ ID NO:1. The term "homology" as used herein can be equated with the term "identity". Relative sequence homology (i.e., sequence identity) can be determined by commercially available computer programs that can calculate the percent  
10 homology between two or more sequences. A typical example of such a computer program is CLUSTAL.

"Substantial homology," where homology indicates sequence identity, means at least 80% sequence identity, as judged by direct sequence alignment and comparison.  
15 "Substantial homology" when assessed by the BLAST algorithm equates to sequences which match with an EXPECT value of at least about 7, e.g., at least about 9, 10, or more. The default threshold for EXPECT in BLAST searching is usually 10.

20 Also included within the scope of the present invention are alleles of afACCase gene. As used herein, an "allele" or "allelic sequence" is an alternative form of afACCase. Alleles result from a mutation, i.e., a change in the nucleotide sequence, and generally produce altered mRNAs or  
25 polypeptides whose structure or function may or may not be altered. Any given gene can have none, one, or more than one allelic form. Common mutational changes which give rise to alleles are generally ascribed to deletions, additions or substitutions of amino acids. Each of these types of changes  
30 can occur alone, or in combination with the others, one or more times in a given sequence.

The ACCase polypeptides of the invention include, but are not limited to, recombinant polypeptides and natural

polypeptides. Also included are nucleic acid sequences that encode forms of ACCase polypeptides in which naturally occurring amino acid sequences are altered or deleted. Preferred nucleic acids encode polypeptides that are soluble  
5 under normal physiological conditions. Also within the invention are nucleic acids encoding fusion proteins in which a portion of the ACCase polypeptide is fused to an unrelated polypeptide (e.g., a marker polypeptide or a fusion partner) to create a fusion protein. For example, the polypeptide can  
10 be fused to a hexa-histidine tag to facilitate purification of bacterially expressed polypeptides, or to a hemagglutinin tag to facilitate purification of polypeptides expressed in eukaryotic cells. The invention also includes, for example, isolated polypeptides (and the nucleic acids that encode  
15 these polypeptides) that include a first portion and a second portion; the first portion includes, e.g., an ACCase polypeptide, and the second portion includes an immunoglobulin constant (Fc) region or a detectable marker.

The fusion partner can be, for example, a polypeptide  
20 that facilitates secretion, e.g., a secretory sequence. Such a fused polypeptide is typically referred to as a preprotein. The secretory sequence can be cleaved by the host cell to form the mature protein. Also within the invention are nucleic acids that encode an ACCase polypeptide fused to a  
25 polypeptide sequence to produce an inactive preprotein. Preproteins can be converted into the active form of the protein by removal of the inactivating sequence.

The invention also includes nucleic acids that hybridize, e.g., under stringent hybridization conditions (as  
30 defined herein) to all or a portion of the nucleotide sequences represented by SEQ ID NO:1, or its complement. The hybridizing portion of the hybridizing nucleic acids is typically at least 15 (e.g., 20, 30, or 50) nucleotides in

length. The hybridizing portion of the hybridizing nucleic acid is at least 60%, e.g., at least 70%, 80%, 95%, or at least 98% or 100%, identical to the sequence of a portion or all of a nucleic acid encoding an ACCase polypeptide or its complement. Hybridizing nucleic acids of the type described herein can be used as a cloning probe, a primer (e.g., a PCR primer), or a diagnostic probe. Nucleic acids that hybridize to the nucleotide sequence represented by SEQ ID NO: 1 are considered "antisense oligonucleotides."

Also useful in the invention are various engineered cells, e.g., transformed host cells, that contain an ACCase nucleic acid described herein. A transformed cell is a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a nucleic acid encoding an ACCase polypeptide. Both prokaryotic and eukaryotic cells are included, e.g., fungi, and bacteria, such as *E. coli*, and the like.

Also useful in the invention are genetic constructs (e.g., vectors and plasmids) that include a nucleic acid of the invention operably linked to a transcription and/or translation sequence to enable expression, e.g., expression vectors. A selected nucleic acid, e.g., a DNA molecule encoding an ACCase polypeptide, is "operably linked" when it is positioned adjacent to one or more sequence elements, e.g., a promoter, which direct transcription and/or translation of the sequence such that the sequence elements can control transcription and/or translation of the selected nucleic acid.

The invention also features purified or isolated polypeptides encoded by the *A. fumigatus* ACCase coding sequence. The terms "protein" and "polypeptide" both refer to any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or

phosphorylation). Thus, the term ACCase polypeptide includes full-length, naturally occurring, isolated ACCase proteins, as well as recombinantly or synthetically produced polypeptides that correspond to the full-length, naturally occurring proteins, or to a portion of the naturally occurring or synthetic polypeptide.

A purified or isolated compound is a composition that is at least 60% by weight the compound of interest, e.g., an ACCase polypeptide or antibody. Preferably the preparation is at least 75% (e.g., at least 90%, 95%, or even 99%) by weight the compound of interest. Purity can be measured by any appropriate standard method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

Preferred ACCase polypeptides include a sequence substantially identical to all or a portion of a naturally occurring *A. fumigatus* ACCase polypeptide, e.g., including all or a portion of the sequences shown in Fig. 1.

Polypeptides "substantially identical" to the ACCase polypeptide sequences described herein have an amino acid sequence that is at least 65% identical to the amino acid sequence of the ACCase polypeptide represented by the SEQ ID NO:2 (measured as described herein). The new polypeptides can also have a greater percentage identity, e.g., 85%, 90%, 95%, or even higher. For purposes of comparison, the length of the reference ACCase polypeptide sequence will generally be at least 16 amino acids, e.g., at least 20 or 25 amino acids.

In the case of polypeptide sequences that are less than 100% identical to a reference sequence, the non-identical positions are preferably, but not necessarily, conservative substitutions for the reference sequence. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine,

and leucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine.

Where a particular polypeptide is said to have a  
5 specific percent identity to a reference polypeptide of a defined length, the percent identity is relative to the reference polypeptide. Thus, a polypeptide that is 50% identical to a reference polypeptide that is 100 amino acids long can be a 50 amino acid polypeptide that is completely  
10 identical to a 50 amino acid long portion of the reference polypeptide. It also might be a 100 amino acid long polypeptide which is 50% identical to the reference polypeptide over its entire length. Of course, other polypeptides also will meet the same criteria.

15 The invention also features purified or isolated antibodies that specifically bind to an *A. fumigatus* ACCase polypeptide. An antibody "specifically binds" to a particular antigen, e.g., an ACCase polypeptide, when it binds to that antigen, but does not recognize and bind to  
20 other molecules in a sample, e.g., a biological sample, that naturally includes an ACCase polypeptide. In addition, an antibody specifically binds to an *A. fumigatus* ACCase polypeptide when it does not substantially bind to ACCase polypeptides from other genres (e.g., *Saccharomyces*),  
25 particularly ACCase polypeptides of an organism to be treated by the methods of the invention (e.g., humans, or domesticated animals).

#### Identifying the *Aspergillus fumigatus* ACCase Gene

30 As shown by the experiments described below, the *Aspergillus fumigatus* ACCase gene is essential for survival. *Aspergillus fumigatus* is available from the ATCC. The *A. fumigatus* ACCase gene was cloned using polymerase chain

reaction technology and degenerate primers based on the *Saccharomyces cerevisiae* ACCase gene. The degenerate primers were used to amplify genomic *Aspergillus fumigatus* DNA using 35 cycles of: 94°C for 1 minute, 40°C for 2 minutes, and 72°C for 3 minutes. The resulting PCR product was subcloned into the pBluescript cloning vector (Stratagene; La Jolla, CA), then sequenced. Based on the resulting sequence, two exact-match primers were created, and the exact-match primers were used to PCR amplify the 5' and 3' halves of the afACCase from an *Aspergillus fumigatus* cDNA library. The cDNA library was made using the vector pYES2 (Invitrogen; Palo Alto, CA). For PCR amplification, one exact-match primer was paired with a primer hybridizing to the 3' sequence of the multiple cloning site of pYES2. The other exact-match primer was paired with a primer hybridizing to the pGAL sequences in pYES2. PCR amplification of the 5' and 3' halves of the ACCase gene was carried out with 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72° for and 2.5 minutes. The resulting PCR products were cloned into the pBluescript vector and sequenced to obtain the cDNA sequence of *Aspergillus fumigatus* ACCase. The entire ACCase open reading frame was subsequently amplified using primers that exactly matched each of (a) the first methionine codon and (b) the stop codon of the ACCase open reading frame. The amplified open reading frame subsequently was cloned into the pCRTOP0 vector (Invitrogen) using TA cloning methods (Invitrogen).

### Identification of ACCase Genes in Additional Fungal Strains

Since the *Aspergillus fumigatus* ACCase gene has been identified, this gene, or fragments thereof, can be used to detect homologous genes in yet other organisms. Fragments of  
5 a nucleic acid (DNA or RNA) encoding an ACCase polypeptide (or sequences complementary thereto) can be used as probes in conventional nucleic acid hybridization assays of various organisms. For example, nucleic acid probes (which typically are 8-30, or usually 15-20, nucleotides in length) can be  
10 used to detect ACCase genes in art-known molecular biology methods, such as Southern blotting, Northern blotting, dot or slot blotting, PCR amplification methods, colony hybridization methods, and the like. Typically, an oligonucleotide probe based on the nucleic acid sequences  
15 described herein, or fragment thereof, is labeled and used to screen a genomic library constructed from mRNA obtained from a fungal strain of interest. A suitable method of labeling involves using polynucleotide kinase to add  $^{32}\text{P}$ -labeled ATP to the oligonucleotide used as the probe. This method is  
20 well known in the art, as are several other suitable methods (e.g., biotinylation and enzyme labeling).

Hybridization of the oligonucleotide probe to the library, or other nucleic acid sample, typically is performed under moderate to high stringency conditions. Nucleic acid  
25 duplex or hybrid stability is expressed as the melting temperature or  $T_m$ , which is the temperature at which a probe dissociates from a target DNA. This melting temperature is used to define the required stringency conditions. If sequences are to be identified that are related and  
30 substantially identical to the probe, rather than identical, then it is useful to first establish the lowest temperature at which only homologous hybridization occurs with a particular concentration of salt (e.g., SSC or SSPE). Then,



assuming that 1% mismatching results in a 1°C decrease in the  $T_m$ , the temperature of the final wash in the hybridization reaction is reduced accordingly (for example, if sequences having  $\geq 95\%$  identity with the probe are sought, the final wash temperature is decreased by 5°C). In practice, the change in  $T_m$  can be between 0.5° and 1.5°C per 1% mismatch.

High stringency conditions are hybridizing at 68°C in 5x SSC/5x Denhardt's solution/1.0% SDS, or in 0.5 M  $\text{NaHPO}_4$  (pH 7.2)/1 mM EDTA/7% SDS, or in 50% formamide/0.25 M  $\text{NaHPO}_4$  (pH 7.2)/0.25 M NaCl/1 mM EDTA/7% SDS; and washing in 0.2x SSC/0.1% SDS at room temperature or at 42°C, or in 0.1x SSC/0.1% SDS at 68°C, or in 40 mM  $\text{NaHPO}_4$  (pH 7.2)/1 mM EDTA/5% SDS at 50°C, or in 40 mM  $\text{NaHPO}_4$  (pH 7.2) 1 mM EDTA/1% SDS at 50°C. Stringent conditions include washing in 3x SSC at 42°C. The parameters of salt concentration and temperature can be varied to achieve the optimal level of identity between the probe and the target nucleic acid. Additional guidance regarding such conditions is available in the art, for example, by Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y.; and Ausubel et al. (eds.), 1995, Current Protocols in Molecular Biology, (John Wiley & Sons, N.Y.) at Unit 2.10.

In one approach, libraries constructed from pathogenic or non-pathogenic fungal strains are screened. For example, such strains can be screened for expression of the ACCase gene of the invention by Northern blot analysis. Upon detection of transcripts of the ACCase gene, libraries can be constructed from RNA isolated from the appropriate strain, utilizing standard techniques well known to those of skill in the art. Alternatively, a total genomic DNA library can be screened using an ACCase gene probe.

New gene sequences can be isolated, for example, by performing PCR using two degenerate oligonucleotide primer

pools designed on the basis of nucleotide sequences within the ACCase gene as depicted herein. The template for the reaction can be DNA obtained from strains known or suspected to express the ACCase gene of the invention. The PCR product  
5 can be subcloned and sequenced.

Synthesis of the various ACCase polypeptides (or an antigenic fragment thereof) for use as antigens, or for other purposes, can be accomplished using any of the various art-known techniques. For example, an ACCase polypeptide, or an  
10 antigenic fragment(s), can be synthesized chemically in vitro, or enzymatically (e.g., by in vitro transcription and translation). Alternatively, the gene can be expressed in, and the polypeptide purified from, a cell (e.g., a cultured cell) by using any of the numerous, available gene expression  
15 systems. For example, the polypeptide antigen can be produced in a prokaryotic host (e.g., *E. coli*) or in eukaryotic cells, such as yeast cells.

Proteins and polypeptides can also be produced in plant cells, if desired. For plant cells, viral expression vectors  
20 (e.g., cauliflower mosaic virus and tobacco mosaic virus) and plasmid expression vectors (e.g., Ti plasmid) are suitable. Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, MD; also, see, e.g., Ausubel et al., *Current Protocols in Molecular*  
25 *Biology*, John Wiley & Sons, New York, 1994). The optimal methods of transformation or transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al., *supra*; expression vehicles can be chosen  
30 from those provided, e.g., in *Cloning Vectors: A Laboratory Manual* (P.H. Pouwels et al., 1985, Supp. 1987). The host cells harboring the expression vehicle can be cultured in conventional nutrient media, adapted as needed for activation.

of a chosen gene, repression of a chosen gene, selection of transformants, or amplification of a chosen gene.

If desired, ACCase polypeptide can be produced as a fusion protein. For example, the expression vector pUR278  
5 (Ruther et al., *EMBO J.*, 2:1791, 1983) can be used to create *lacZ* fusion proteins. The art-known pGEX vectors can be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion  
10 proteins are soluble and can be easily purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

15 In an exemplary expression system, a baculovirus such as *Autographa californica* nuclear polyhedrosis virus (AcNPV), which grows in *Spodoptera frugiperda* cells, can be used as a vector to express foreign genes. A coding sequence encoding an ACCase polypeptide can be cloned into a non-essential  
20 region (for example the polyhedrin gene) of the viral genome and placed under control of a promoter, e.g., the polyhedrin promoter or an exogenous promoter. Successful insertion of a gene encoding an ACCase polypeptide can result in inactivation of the polyhedrin gene and production of non-  
25 occluded recombinant virus (i.e., virus lacking the proteinaceous coat encoded by the polyhedrin gene). These recombinant viruses are then typically used to infect insect cells (e.g., *Spodoptera frugiperda* cells) in which the inserted gene is expressed (see, e.g., Smith et al., *J.*  
30 *Virol.*, 46:584, 1983; Smith, U.S. Patent No. 4,215,051). If desired, mammalian cells can be used in lieu of insect cells, provided that the virus is engineered such that the gene

encoding the ACCase polypeptide is placed under the control of a promoter that is active in mammalian cells.

In mammalian host cells, a number of viral-based expression systems can be utilized. When an adenovirus is  
5 used as an expression vector, the nucleic acid sequence encoding the ACCase polypeptide can be ligated to an adenovirus transcription/ translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene can then be inserted into the adenovirus genome  
10 by *in vitro* or *in vivo* recombination. Insertion into a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing an ACCase gene product in infected hosts (see, e.g., Logan, Proc. Natl. Acad. Sci. USA, 81:3655, 1984).

15 Specific initiation signals can be required for efficient translation of inserted nucleic acid sequences. These signals include the ATG initiation codon and adjacent sequences. In general, exogenous translational control signals, including, perhaps, the ATG initiation codon, should  
20 be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire sequence. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic.  
25 The efficiency of expression can be enhanced by the inclusion of appropriate transcription enhancer elements, or transcription terminators (Bittner et al., *Methods in Enzymol.*, 153:516, 1987).

The ACCase polypeptide can be expressed individually or  
30 as a fusion with a heterologous polypeptide, such as a signal sequence or other polypeptide having a specific cleavage site at the N-and/or C-terminus of the protein or polypeptide. The heterologous signal sequence selected should be one that

is recognized and processed, i.e., cleaved by a signal peptidase, by the host cell in which the fusion protein is expressed.

A host cell can be chosen that modulates the expression  
5 of the inserted sequences, or modifies and processes the gene product in a specific, desired fashion. Such modifications and processing (e.g., cleavage) of protein products can facilitate optimal functioning of the protein. Various host cells have characteristic and specific mechanisms for post-  
10 translational processing and modification of proteins and gene products. Appropriate cell lines or host systems familiar to those of skill in the art of molecular biology can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end,  
15 eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, and phosphorylation of the gene product can be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and choroid  
20 plexus cell lines.

If desired, the ACCase polypeptide can be produced by a stably-transfected mammalian cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public, see, e.g., Pouwels et al. (supra);  
25 methods for constructing such cell lines are also publicly known, e.g., in Ausubel et al. (supra). In one example, DNA encoding the protein is cloned into an expression vector that includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the gene encoding  
30 the afACCase polypeptide into the host cell chromosome is selected for by including 0.01-300  $\mu$ M methotrexate in the cell culture medium (as described in Ausubel et al., supra).

This dominant selection can be accomplished in most cell types.

Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene. Methods for  
5 selecting cell lines bearing gene amplifications are described in Ausubel et al. (supra); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing  
expression vectors commonly used for this purpose include  
10 pCVSEII-DHFR and pAdD26SV(A) (described in Ausubel et al., supra).

A number of other selection systems can be used, including but not limited to, herpes simplex virus thymidine kinase genes, hypoxanthine-guanine phosphoribosyl-transferase  
15 genes, and adenine phosphoribosyltransferase genes, which can be employed in *tk*, *hgp<sub>rt</sub>*, or *ap<sub>rt</sub>* cells, respectively. In addition, *gpt*, which confers resistance to mycophenolic acid (Mulligan et al., *Proc. Natl. Acad. Sci. USA*, 78:2072, 1981); *neo*, which confers resistance to the aminoglycoside G-418  
20 (Colberre-Garapin et al., *J. Mol. Biol.*, 150:1, 1981); and *hygro*, which confers resistance to hygromycin (Santerre et al., *Gene*, 30:147, 1981), can be used.

Alternatively, any fusion protein can be purified by utilizing an antibody or other molecule that specifically  
25 bind to the fusion protein being expressed. For example, a system described in Janknecht et al., *Proc. Natl. Acad. Sci. USA*, 88:8972 (1981), allows for the ready purification of non-denatured fusion proteins expressed in human cell lines. In this system, the gene of interest is subcloned into a  
30 vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded

onto Ni<sup>2+</sup> nitriloacetic acid-agarose columns, and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

Alternatively, an ACCase polypeptide, or a portion thereof, can be fused to an immunoglobulin Fc domain. Such a fusion protein can be purified using a protein A column, for example. Moreover, such fusion proteins permit the production of a chimeric form of an ACCase polypeptide having increased stability *in vivo*.

Once the recombinant ACCase polypeptide is expressed, it can be isolated (i.e., purified). Secreted forms of the polypeptides can be isolated from cell culture media, while non-secreted forms must be isolated from the host cells. Polypeptides can be isolated by affinity chromatography. For example, an anti-ACCase antibody (e.g., produced as described herein) can be attached to a column and used to isolate the protein. Lysis and fractionation of cells harboring the protein prior to affinity chromatography can be performed by standard methods (see, e.g., Ausubel et al., supra).

Alternatively, a fusion protein can be constructed and used to isolate an ACCase polypeptide (e.g., an ACCase-maltose binding fusion protein, an ACCase- $\alpha$ -galactosidase fusion protein, or an ACCase-trpE fusion protein; see, e.g., Ausubel et al., supra; New England Biolabs Catalog, Beverly, MA).

The recombinant protein can, if desired, be further purified, e.g., by high performance liquid chromatography using standard techniques (see, e.g., Fisher, *Laboratory Techniques In Biochemistry And Molecular Biology*, eds., Work and Burdon, Elsevier, 1980).

Given the amino acid sequences described herein, polypeptides useful in practicing the invention, particularly fragments of afACCase, can be produced by standard chemical synthesis (e.g., by the methods described in *Solid Phase*

*Peptide Synthesis*, 2nd ed., The Pierce Chemical Co., Rockford, IL, 1984) and used as antigens, for example.

#### Antibodies

5       The ACCase polypeptides (or antigenic fragments or analogs of such polypeptides) can be used to raise antibodies useful in the invention, and such polypeptides can be produced by recombinant or peptide synthetic techniques (see, e.g., *Solid Phase Peptide Synthesis*, supra; Ausubel et al.,  
10 supra). In general, the polypeptides can be coupled to a carrier protein, such as KLH, as described in Ausubel et al., supra, mixed with an adjuvant, and injected into a host mammal. A "carrier" is a substance that confers stability on, and/or aids or enhances the transport or immunogenicity  
15 of, an associated molecule. Antibodies can be purified, for example, by affinity chromatography methods in which the polypeptide antigen is immobilized on a resin.

In particular, various host animals can be immunized by injection of a polypeptide of interest. Examples of suitable  
20 host animals include rabbits, mice, guinea pigs, and rats. Various adjuvants can be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete adjuvant), adjuvant mineral gels such as aluminum hydroxide, surface  
25 active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from  
30 the sera of the immunized animals.

Antibodies useful in the invention include monoclonal antibodies, polyclonal antibodies, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')<sub>2</sub>



fragments, and molecules produced using a Fab expression library.

Monoclonal antibodies (mAbs), which are homogeneous populations of antibodies to a particular antigen, can be prepared using ACCase, and standard hybridoma technology (see, e.g., Kohler et al., *Nature*, 256:495, 1975; Kohler et al., *Eur. J. Immunol.*, 6:511, 1976; Kohler et al., *Eur. J. Immunol.*, 6:292, 1976; Hammerling et al., In Monoclonal Antibodies and T Cell Hybridomas, Elsevier, NY, 1981; Ausubel et al., supra).

In particular, monoclonal antibodies can be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture, such as those described in Kohler et al., *Nature*, 256:495, 1975, and U.S. Patent No. 4,376,110; the human B-cell hybridoma technique (Kosbor et al., *Immunology Today*, 4:72, 1983; Cole et al., *Proc. Natl. Acad. Sci. USA*, 80:2026, 1983); and the EBV-hybridoma technique (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96, 1983). Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD, and any subclass thereof. The hybridomas producing the mAbs of this invention can be cultivated in vitro or in vivo.

Once produced, polyclonal or monoclonal antibodies are tested for specific recognition of *A. fumigatus* ACCase in an immunoassay, such as a Western blot or immunoprecipitation analysis using standard techniques, e.g., as described in Ausubel et al., supra. Antibodies that specifically bind to the ACCase polypeptide, or conservative variants are useful in the invention. For example, such antibodies can be used in an immunoassay to detect an ACCase polypeptide in pathogenic or non-pathogenic strains of fungi.

Preferably, antibodies of the invention are produced using fragments of ACCase that appear likely to be antigenic, by criteria such as high frequency of charged residues. In one specific example, such fragments are generated by  
5 standard techniques of PCR, and are then cloned into the pGEX expression vector (Ausubel et al., supra). Fusion proteins are expressed in *E. coli* and purified using a glutathione agarose affinity matrix as described in Ausubel, et al., supra.

10 If desired, several (e.g., two or three) fusions can be generated for each protein, and each fusion can be injected into at least two rabbits. Antisera can be raised by injections in a series, typically including at least three booster injections. Typically, the antisera is checked for  
15 its ability to immunoprecipitate a recombinant ACCase polypeptide, or unrelated control proteins, such as glucocorticoid receptor, chloramphenicol acetyltransferase, or luciferase.

Techniques developed for the production of "chimeric  
20 antibodies" (Morrison et al., *Proc. Natl. Acad. Sci.*, 81:6851, 1984; Neuberger et al., *Nature*, 312:604, 1984; Takeda et al., *Nature*, 314:452, 1984) can be used to splice the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody  
25 molecule of appropriate biological activity. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

30 Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; and U.S. Patents 4,946,778 and 4,704,692) can be adapted to produce single chain antibodies against an ACCase polypeptide.

Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments that recognize and bind to specific epitopes can be generated by known techniques. For example, such fragments can include but are not limited to F(ab')<sub>2</sub> fragments, which can be produced by pepsin digestion of the antibody molecule, and Fab fragments, which can be generated by reducing the disulfide bridges of F(ab')<sub>2</sub> fragments.

10 Alternatively, Fab expression libraries can be constructed (Huse et al., *Science*, 246:1275, 1989) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Polyclonal and monoclonal antibodies that specifically bind to an ACCase polypeptide can be used, for example, to detect expression of ACCase in another strain of fungi. For example, an ACCase polypeptide can be detected in conventional immunoassays of fungal cells or extracts. Examples of suitable assays include, without limitation,

20 Western blotting, ELISAs, radioimmune assays, and the like.

#### Assay for Antifungal Agents

The invention provides a method for identifying an antifungal agent(s). Although the inventor is not bound by any particular theory as to the biological mechanism involved, the new antifungal agents are thought to inhibit specifically (1) the function of the ACCase polypeptide or (2) expression of the ACCase gene. In preferred methods, screening for antifungal agents is accomplished by

30 identifying those compounds (e.g., small organic molecules) that inhibit the activity of an ACCase polypeptide or the expression of an ACCase gene. Because ACCase is essential for the survival of *A. fumigatus*, compounds that inhibit

ACCCase in such assays are expected to be antifungal agents and can be further tested, if desired, in conventional susceptibility assays.

In various suitable methods, screening for antifungal agents is accomplished by (i) identifying those compounds that bind to ACCCase and (ii) further testing such compounds for their ability to inhibit fungal growth *in vitro* or *in vivo*.

Specific binding of a test compound to a polypeptide can be detected, for example, *in vitro* by reversibly or irreversibly immobilizing the test compound(s) on a substrate, e.g., the surface of a well of a 96-well polystyrene microtitre plate. Methods for immobilizing polypeptides and other small molecules are well known in the art. For example, the microtitre plates can be coated with an ACCCase polypeptide by adding the polypeptide in a solution (typically, at a concentration of 0.05 to 1 mg/ml in a volume of 1-100  $\mu$ l) to each well, and incubating the plates at room temperature to 37°C for 0.1 to 36 hours. Polypeptides that are not bound to the plate can be removed by shaking the excess solution from the plate, and then washing the plate (once or repeatedly) with water or a buffer. Typically, the polypeptide is in water or a buffer. The plate is then washed with a buffer that lacks the bound polypeptide. To block the free protein-binding sites on the plates, the plates are blocked with a protein that is unrelated to the bound polypeptide. For example, 300  $\mu$ l of bovine serum albumin (BSA) at a concentration of 2 mg/ml in Tris-HCl is suitable. Suitable substrates include those substrates that contain a defined cross-linking chemistry (e.g., plastic substrates, such as polystyrene, styrene, or polypropylene substrates from Corning Costar Corp. (Cambridge, MA), for example). If desired, a beaded particle, e.g., beaded

agarose or beaded sepharose, can be used as the substrate. ACCase is then added to the coated plate and allowed to bind to the test compound (e.g., at 37°C for 0.5-12 hours). The plate then is rinsed as described above.

5 Binding of the test compound to ACCase can be detected by any of a variety of art-known methods. For example, an antibody that specifically binds to an ACCase polypeptide can be used in an immunoassay. If desired, the antibody can be labeled (e.g., fluorescently or with a radioisotope) and  
10 detected directly (see, e.g., West and McMahon, *J. Cell Biol.* 74:264, 1977). Alternatively, a second antibody can be used for detection (e.g., a labeled antibody that binds to the Fc portion of an anti-YphC antibody). In an alternative detection method, the ACCase polypeptide is labeled, and the  
15 label is detected (e.g., by labeling an ACCase polypeptide with a radioisotope, fluorophore, chromophore, or the like). In still another method, the ACCase polypeptide is produced as a fusion protein with a protein that can be detected optically, e.g., green fluorescent protein (which can be  
20 detected under UV light). In an alternative method, the polypeptide can be produced as a fusion protein with an enzyme having a detectable enzymatic activity, such as horse radish peroxidase, alkaline phosphatase, -galactosidase, or glucose oxidase. Genes encoding all of these enzymes have  
25 been cloned and are available for use by those of skill in the art. If desired, the fusion protein can include an antigen, and such an antigen can be detected and measured with a polyclonal or monoclonal antibody using conventional methods. Suitable antigens include enzymes (e.g., horse  
30 radish peroxidase, alkaline phosphatase, and -galactosidase) and non-enzymatic polypeptides (e.g., serum proteins, such as BSA and globulins, and milk proteins, such as caseins).

In various *in vivo* methods for identifying polypeptides that bind to ACCase, the conventional two-hybrid assays of protein/protein interactions can be used (see e.g., Chien et al., *Proc. Natl. Acad. Sci. USA*, **88**:9578, 1991; Fields et al., U.S. Pat. No. 5,283,173; Fields and Song, *Nature*, **340**:245, 1989; Le Douarin et al., *Nucleic Acids Research*, **23**:876, 1995; Vidal et al., *Proc. Natl. Acad. Sci. USA*, **93**:10315-10320, 1996; and White, *Proc. Natl. Acad. Sci. USA*, **93**:10001-10003, 1996). Generally, the two-hybrid methods involve *in vivo* reconstitution of two separable domains of a transcription factor. One fusion protein contains the ACCase polypeptide fused to either a transactivator domain or DNA binding domain of a transcription factor (e.g., of Gal4). The other fusion protein contains a test polypeptide fused to either the DNA binding domain or a transactivator domain of a transcription factor. Once brought together in a single cell (e.g., a yeast cell or mammalian cell), one of the fusion proteins contains the transactivator domain and the other fusion protein contains the DNA binding domain. Therefore, binding of the ACCase polypeptide to the test polypeptide (i.e., candidate antifungal agent) reconstitutes the transcription factor. Reconstitution of the transcription factor can be detected by detecting expression of a gene (i.e., a reporter gene) that is operably linked to a DNA sequence that is bound by the DNA binding domain of the transcription factor. Kits for practicing various two-hybrid methods are commercially available (e.g., from Clontech; Palo Alto, CA).

The methods described above can be used for high throughput screening of numerous test compounds to identify candidate antifungal (or anti-fungal) agents. Having identified a test compound as a candidate antifungal agent, the candidate antifungal agent can be further tested for

inhibition of fungal growth *in vitro* or *in vivo* (e.g., using an animal, e.g., rodent, model system) if desired. Using other, art-known variations of such methods, one can test the ability of a nucleic acid (e.g., DNA or RNA) used as the test  
5 compound to bind to ACCase.

*In vitro*, further testing can be accomplished by means known to those in the art such as an enzyme inhibition assay or a whole-cell fungal growth inhibition assay. For example, an agar dilution assay identifies a substance that inhibits  
10 fungal growth. Microtiter plates are prepared with serial dilutions of the test compound, adding to the preparation a given amount of growth substrate, and providing a preparation of fungi. Inhibition of fungal growth is determined, for example, by observing changes in optical densities of the  
15 fungal cultures.

Inhibition of fungal growth is demonstrated, for example, by comparing (in the presence and absence of a test compound) the rate of growth or the absolute growth of fungal cells. Inhibition includes a reduction in the rate of growth  
20 or absolute growth by at least 20%. Particularly potent test compounds can further reduce the growth rate (e.g., by at least 25%, 30%, 40%, 50%, 75%, 80%, or 90%).

Animal (e.g., rodent such as murine) models of fungal infections are known to those of skill in the art, and such  
25 animal model systems are accepted for screening antifungal agents as an indication of their therapeutic efficacy in human patients. In a typical *in vivo* assay, an animal is infected with a pathogenic strain of fungi, e.g., by inhalation of fungi, and conventional methods and criteria  
30 are used to diagnose the mammal as being afflicted with a fungal infection. The candidate antifungal agent then is administered to the mammal at a dosage of 1-100 mg/kg of body weight, and the mammal is monitored for signs of amelioration

of disease. Alternatively, the test compound can be administered to the mammal prior to infecting the mammal with the fungi, and the ability of the treated mammal to resist infection is measured. Of course, the results obtained in  
5 the presence of the test compound should be compared with results in control animals, which are not treated with the test compound. Administration of candidate antifungal agents to the mammal can be carried out as described below, for example.

10

#### Pharmaceutical Formulations

Treatment includes administering a pharmaceutically effective amount of a composition containing an antifungal agent to a subject in need of such treatment, thereby  
15 inhibiting fungal growth in the subject. Such a composition typically contains from about 0.1 to 90% by weight (such as 1 to 20% or 1 to 10%) of an antifungal agent of the invention in a pharmaceutically acceptable carrier.

Solid formulations of the compositions for oral  
20 administration can contain suitable carriers or excipients, such as corn starch, gelatin, lactose, acacia, sucrose, microcrystalline cellulose, kaolin, mannitol, dicalcium phosphate, calcium carbonate, sodium chloride, or alginic acid. Disintegrators that can be used include, without  
25 limitation, micro-crystalline cellulose, corn starch, sodium starch glycolate and alginic acid. Tablet binders that can be used include acacia, methylcellulose, sodium carboxymethylcellulose, polyvinylpyrrolidone (Povidone), hydroxypropyl methylcellulose, sucrose, starch, and  
30 ethylcellulose. Lubricants that can be used include magnesium stearates, stearic acid, silicone fluid, talc, waxes, oils, and colloidal silica.



Liquid formulations of the compositions for oral administration prepared in water or other aqueous vehicles can contain various suspending agents such as methylcellulose, alginates, tragacanth, pectin, kelgin, 5 carrageenan, acacia, polyvinylpyrrolidone, and polyvinyl alcohol. The liquid formulations can also include solutions, emulsions, syrups and elixirs containing, together with the active compound(s), wetting agents, sweeteners, and coloring and flavoring agents. Various liquid and powder formulations 10 can be prepared by conventional methods for inhalation into the lungs of the mammal to be treated.

Injectable formulations of the compositions can contain various carriers such as vegetable oils, dimethylacetamide, dimethylformamide, ethyl lactate, ethyl carbonate, isopropyl 15 myristate, ethanol, polyols (glycerol, propylene glycol, liquid polyethylene glycol, and the like). For intravenous injections, water soluble versions of the compounds can be administered by the drip method, whereby a pharmaceutical formulation containing the antifungal agent and a 20 physiologically acceptable excipient is infused. Physiologically acceptable excipients can include, for example, 5% dextrose, 0.9% saline, Ringer's solution or other suitable excipients. Intramuscular preparations, a sterile formulation of a suitable soluble salt form of the compounds 25 can be dissolved and administered in a pharmaceutical excipient such as Water-for-Injection, 0.9% saline, or 5% glucose solution. A suitable insoluble form of the compound can be prepared and administered as a suspension in an aqueous base or a pharmaceutically acceptable oil base, such 30 as an ester of a long chain fatty acid, (e.g., ethyl oleate).

A topical semi-solid ointment formulation typically contains a concentration of the active ingredient from about 1 to 20%, e.g., 5 to 10% in a carrier such as a

pharmaceutical cream base. Various formulations for topical use include drops, tinctures, lotions, creams, solutions, and ointments containing the active ingredient and various supports and vehicles.

- 5       The optimal percentage of the antifungal agent in each pharmaceutical formulation varies according to the formulation itself and the therapeutic effect desired in the specific pathologies and correlated therapeutic regimens. Appropriate dosages of the antifungal agents can be
- 10 determined by those of ordinary skill in the art of medicine by monitoring the mammal for signs of disease amelioration or inhibition, and increasing or decreasing the dosage and/or frequency of treatment as desired. The optimal amount of the antifungal compound used for treatment of conditions caused
- 15 by or contributed to by fungal infection depends upon the manner of administration, the age and the body weight of the subject, and the condition of the subject to be treated. Generally, the antifungal compound is administered at a dosage of 1 to 100 mg/kg of body weight, and typically at a
- 20 dosage of 1 to 10 mg/kg of body weight.

#### Experiments

A detailed example of the preparation of ACCase follows.

#### 25 Materials and Methods

The following reagents and equipment were used. Similar reagents and equipment can be substituted for those listed herein.

<u>REAGENTS</u>		<u>Supplier</u>
30 1-Butanol		VWR Scientific EM-BX1780-5
DTT		VWR Scientific
dithiothreitol		IB21040
35		

	Bacto-Peptone	VWR Scientific DF-0118-17
5	Bacto-Yeast Extract	VWR Scientific DF-0127-17
	Bacto-Agar	VWR Scientific DF-0140-01
10	Bacto-Yeast Nitrogen Base w/o amino acids	Difco 0919-15
	Dimethylsulphoxide (DMSO)	VWR Scientific D-128
15	EGTA (ethylene glycol-bis( -aminoethyl ether) -N,N,N'N'-tetraacetic acid)	Sigma Chemical Co E-0396
20	Myristoyl CoA	Sigma Chemical Co M-9142
	Glucose	Sigma Chemical Co G-5767
25	Glycerol	VWR Scientific JT2136-1
	Glycine	VWR Scientific EM-GX0205MB-5
30	Hydrochloric acid, concentrated	VWR Scientific JT9535-33
35	Magnesium chloride hexahydrate	VWR Scientific JT2444-1
	EDTA (ethylenediamine tetraacetic acid, disodium salt dihydrate)	VWR Scientific EM-EX0539MB-4
40	MicroScint™ 40 Liquid Scintillation Cocktail	Packard 6013641
	Micro™ Concentrated Cleaning Solution	VWR Scientific

		21830-410
	Ammonium Sulfate	VWR Scientific
5		EM-1209-2
	D-Biotin, ImmunoPure	Pierce
		29129
10	Biotinylated BSA, ImmunoPure (Bovine Serum Albumin)	Pierce
		29130
	PMSF	Boehringer
	Mannheim	
15	(Phenylmethanesulfonyl Fluoride)	837 091
	leupeptin	Boehringer
	Mannheim	
		1017 128
20	antipain dihydrochloride	Boehringer
	Mannheim	
		1004 646
	APMSF	Boehringer
25	Mannheim	
	[(4-amidinophenyl)-methanesulfonyl fluoride hydrochloride monohydrate]	917 575
	pepstatin	Boehringer
30		Mannheim
		B1359 053
	Immunopure™ Monomeric Avidin/Agarose	Pierce
35		20228
	BupH™ Phosphate Buffered Saline (0.1M phosphate, 0.15M NaCl, pH 7.2)	Pierce
		28372
40	Sodium Fluoride	VWR Scientific
		JT3688-1
	Potassium Chloride	VWR Scientific
		JT3040-1
	Sucrose	VWR Scientific

		JT4072-5
	TRIS	VWR Scientific
	Tris (hydroxymethyl) aminomethane	EM-TX1530MB-2
5	Sodium Bicarbonate	VWR Scientific
		JT3506-1
	ATP	Sigma Chemical Co
10	Adenosine 5'-triphosphate, disodium salt	A-7699
	Acetyl Coenzyme A	Sigma Chemical Co
	Sodium Salt	A-2056
15	Sodium [ <sup>14</sup> C] bicarbonate*	Amersham Life
	Science	
	2mCi/ml	CFA 3
20	<u>EQUIPMENT LIST</u>	
	10 1 Disposable Loops for inoculating	VWR Scientific
		60872-406
25	10L fermenter	New Brunswick
		Scientific
	fermenter	Microferm
	Bead-Beater complete package	Biospec Products,
30		Bartlesville, OK
	0.5mm diameter glass beads	Biospec Products
		Bartlesville OK
35	Nalgene screw-cap polypropylene	VWR Scientific
	centrifuge tubes 50 ml capacity	21009-386
	Beckman J2-MI high-speed centrifuge	
40	Beckman JA-17 high-speed centrifuge rotor	
	Beckman J6-MI centrifuge	
	Beckman JS-4.0 rotor	

Beckman Optima™ ultracentrifuge

Beckman SW41-Ti ultracentrifuge rotor

5 Beckman Ultra-Clear centrifuge tubes  
14x89 mm

10 Potter-Elvehjem Tissue Grinders, Wheaton VWR Scientific  
5ml 62400-722

pH meter fitted with Tris compatible electrode

15 Finnpiquette 5-50 l 12 channel pipetman

Vortex Genie 2 VWR Scientific  
58815-178

20 250ml Sterilization Filter Unit, 0.2 m VWR Scientific  
Cellulose Nitrate (Nalgene) 28199-111

1L Sterilization Filter Unit, 0.2 m VWR Scientific  
Cellulose Nitrate (Nalgene) 28199-268

25 250ml Erlenmeyer Flask VWR Scientific  
(Kimax) 28140-544

Gilson P2, P100, P1000 pipetmen with tips Rainin

30 Falcon 15 ml screw cap conical tube VWR Scientific  
21008-918

Falcon 50 ml screw cap conical tube VWR Scientific  
21008-951

35 100x15mm Sterile Disposable Petri Dishes VWR Scientific  
(Falcon) 25380-069

250ml screw-cap disposable centrifuge VWR Scientific  
40 tubes (Corning) 21008-771

2 ml cryogenic vials VWR Scientific  
(Nalgene) 66008-728

- |   |   |
|---|---|
| 4ml cryogenic vials, round bottom<br>(Corning)                          | VWR Scientific<br>66021-946                           |
| 5 TopCount™ Scintillation Counter                                       | Packard   |
| OptiPlate™<br>Opaque 96-well microtiter plates for TopCount             | Packard<br>6005190                                    |
| 10 EM ColorpHast pH paper, narrow range<br>2.5-4.5<br>4.0-7.0<br>6.5-10 | VWR Scientific<br>EM-9581-3<br>EM-9582-3<br>EM-9583-3 |
| 15 10L Polypropylene Carboy with spigot<br>and handles                  | VWR-Scientific<br>(Nalgene)<br>16101-404              |
| Ranson Slide Warmer (LabLine)   | VWR Scientific  |
| 20 [3.5Hx28Wx8D"]   | 15160-797   |
| MultiDrop 96-well dispenser   | Titertec™   |
| Quadra 96, Model 320 Automatic Pipettor                                 | Tomtec™, Inc.   |
| 25  |   |

STOCK SOLUTIONS

- 30 1M Tris, pH 7.5 [add 500 ml dI H<sub>2</sub>O to 121.4g solid Tris base; adjust pH to 7.5 with concentrated HCl; make up to 1L with dI H<sub>2</sub>O]
- 35 10N NaOH [add 90 ml pre-chilled dI H<sub>2</sub>O to 40g pellets; stir vigorously on ice; make up to 100ml; store in screw-cap polypropylene bottle not glass]
- 40 0.5 M Na<sub>2</sub> EDTA, pH 8.0 [46.5g solid; suspend in 200ml dI H<sub>2</sub>O; pH; pH with 10N NaOH; make up to 250ml; stir vigorously until dissolved]
- 1M MgCl<sub>2</sub> [dissolve 51g MgCl<sub>2</sub> 6H<sub>2</sub>O in 250ml dI H<sub>2</sub>O]

- 1M Glycine, pH 2.8 [dissolve 18.75g Glycine in 150ml dI H<sub>2</sub>O;  
pH with concentrated HCl; make  
up to 250ml; filter through 0.2 m using Nalgene 250ml  
Sterilization filter unit]
- 5 1M NaHCO<sub>3</sub> [dissolve 8.4g solid NaHCO<sub>3</sub> in 90ml dI H<sub>2</sub>O with  
vigorous stirring; make up to 100 ml]
- 10 1M DTT [dissolve 2.31g solid DTT in 15ml dI H<sub>2</sub>O dispense in  
0.75ml aliquots and store at -20°C]
- 15 200mM ATP [dissolve 13.2g in 100 ml dI H<sub>2</sub>O; adjust pH to 7  
with 10N NaOH (use narrow range pH paper; add 2ml base, check  
pH, then 100 l increment); make up to 200ml; store as 40ml  
aliquots at -80°C in 50ml Falcon Tubes]
- 15mM Acetyl CoA [dissolve 5\*100mg Na<sub>2</sub> (AcCoA) in 40 ml dI  
H<sub>2</sub>O; store as 10ml aliquots at -80°C]
- 20 3mM Myristoyl CoA [dissolve 5mg myristoyl CoA in 1.7 ml DMSO  
Store at -20°C]

#### WORKING SOLUTIONS

- 25 (A) UltraLink™ Monomeric Avidin Column Equilibration
- Phosphate Buffered Saline (PBS)**  
0.1M phosphate, 0.15M NaCl, pH 7.2 [prepared using Pierce  
30 PubH™ mixture]
- Regeneration Buffer**  
0.1M Glycine, pH 2.8 [30ml 1M Stock, made up to 300ml]
- 35 **Biotin Blocking and Elution Buffer**  
2mM Biotin in PBS [48.9mg D-biotin, made up to 100ml with  
PBS]
- 40 (B) Cell Growth
- |                                |                   |
|--------------------------------|-------------------|
| YPD growth medium (for plates) | [to make 1 liter] |
| 1% yeast extract               | [10g]             |
| 2% peptone                     | [20g]             |



2% glucose [20g]  
2% agar [20g]

Autoclave, cool to 60°C, dispense into disposable plastic  
5 petri dishes in flow hood. Store plates at 4°C in plastic bag.

**20% (w/v) Glucose** [to make 1 liter]  
Dissolve 200g glucose in 1 L dI H<sub>2</sub>O, autoclave.

10

**10X SD + uridine growth medium** [to make 1 liter]  
67g yeast nitrogen base w/o amino acids  
300mg uridine

15 Make up to 1L with 20% (w/v) glucose solution; Filter  
sterilize using 0.2 micron filter bottle.

(C) Enzyme Preparation

20

**Cell breakage buffer (Buffer A):** [to make 1L]  
100mM Tris, pH 7.5 [100ml 1M stock]  
100mM Sodium Fluoride [4.2g solid]  
1mM EDTA [2ml 0.5M stock]  
25 1mM EGTA [380mg solid]  
10mM DTT [1.54g solid]  
0.25M sucrose [86g solid]  
dI H<sub>2</sub>O to 1L.

Store at 4°C; Prepare Fresh

30

**Column Loading buffer (Buffer B):** [ to make 500ml]  
50mM Tris, pH 7.5 [25ml 1M stock]  
10mM DTT [0.77g solid]  
35 0.5M KCl [18.6g solid]  
2mM EDTA [2ml 0.5M stock]  
50ml glycerol  
dI H<sub>2</sub>O to 500ml.

Store at 4°C; Prepare Fresh

40

**Enzyme Storage Buffer** [to make 1L]  
50mM Tris, pH 7.0 [50ml 1M Stock]  
1mM EDTA [2ml 0.5M stock]  
5mM DTT [0.77g solid]

Make up to 500ml with dI H<sub>2</sub>O; Add 500ml glycerol. Store at 4°C; Prepare Fresh.

## 5 Protease Inhibitors

Inhibitor stock	Concentration	Stock Concentration in buffer	Concentration Amount /100ml Buffer
10 PMSF	170 g/ml	100mg/ml*	170 l
leupeptin	0.5 g/ml	1mg/ml	50 l
pepstatin	0.7 g/ml	1mg/ml*	70 l
antipain	50 g/ml	10mg/ml	500 l
APMSF	40 g/ml	10mg/ml	400 l
15		*In 100% methanol	

## (D) High-Throughput Screen

20 20% (v/v) DMSO [Make up 1L]

ACCCase Assay Buffer minus DTT [to make up 1L]  
 83.33mM Tris, pH 7.5 [83.3ml 1M Stock]  
 8.33mM MgCl<sub>2</sub> [8.33ml 1M Stock]

25 dI H<sub>2</sub>O [up to 1L]

ACCCase Assay Buffer [to make up 250ml, sufficient for 25 plates] [Add 833 l 1M DTT to 250ml ACCCase Assay Buffer minus DTT (3.33mM DTT final concentration)]

30

5X Cold Substrate Buffer  
 [to make up 390ml, sufficient for 150 plates]  
 20.5mM ATP [40ml 200mM ATP, pH 7]  
 5.13mM NaHCO<sub>3</sub> [2ml 1M NaHCO<sub>3</sub>]

35 384 M Acetyl CoA [10ml 15mM Acetyl CoA]  
 dI H<sub>2</sub>O [up to 390ml]  
 Store as 39ml aliquots at -80°C

## 5X Radiometric Substrate Buffer

40 [to make up 40ml, sufficient for 15 plates]  
 5X Cold Substrate Buffer [39ml; see above]  
 NaH[<sup>14</sup>C]O<sub>3</sub> [1ml 2mCi/ml Stock]  
 Prepare immediately before addition to screening plates, as <sup>14</sup>C bicarbonate exchanges with atmospheric CO<sub>2</sub>

- Stop Solution (2N HCl) [to make 10L]  
[add 1.67L concentrated HCl to 7L pre-chilled dI H<sub>2</sub>O on ice slowly, with stirring; Make up to 10L with dI H<sub>2</sub>O]  
5 Store in a 10L Nalgene Carboy at Room temp.

#### METHODS

##### 10 (A) Preparation of Enzyme from *A. fumigatus*.

###### (1) Growth of cells:

A strain of *A. fumigatus* was stored as a glycerol stock  
15 in 2ml cryovials at -80°C. Cells were streaked out onto YPD plates using a 10 l Loop and incubated for 24-48 hours at 30°C.

The strain of *A. fumigatus* from plates was inoculated into 50ml of SD+uridine and incubated overnight, with  
20 shaking, at 30°C. Cells were subcultured into 250ml SD+uridine and shaken at 30°C for 8-9 hours. This culture was used to inoculate (in parallel) two 10L fermenters containing SD+uridine to a starting OD<sub>600</sub> of 0.01 to 0.015. [Fermenters were autoclaved containing 9L of dI water, and 1L  
25 of 10X SD+uridine was added 30 minutes before inoculation.] The cells were grown overnight at 30°C to a final OD<sub>600</sub> of 2.0-2.5 (mid-exponential phase) and harvested by centrifugation (J6-MI centrifuge, JS-4.0 rotor, 3000rpm for 10 minutes). The fermentation takes about 17 hours, and the  
30 harvest time can be calculated (accuracy ± half hour) based on the inoculation OD, and lag and doubling times. In a typical run, fermenters were inoculated at 6pm and harvested at 10am the following day. Lag time was ≤ 15 minutes and doubling time was 2 hours. The second fermenter was  
35 inoculated to a slightly lower OD, such that cells were ready for harvesting about 45 minutes to 1 hour after the first was

ready (to allow for the time it takes to harvest the first). About 160g of packed cells were obtained from two 10L fermenters.

After harvesting in Corning 250ml disposable centrifuge tubes, cells were immediately frozen at -80°C and stored until processing.

(2) Lysis of cells and preparation of 40% ammonium sulfate precipitate

10 All operations were performed at 4°C. Protease inhibitors were added to Buffer A (cell breakage buffer) and one volume of this mixture was added to one volume of packed cells (75g cells yields 150ml of suspension; sufficient to fill the bead-beater chamber). The cells were resuspended by  
15 brief vortexing, and the cell suspension was transferred to the bead-beater chamber (already half-filled with prewetted glass beads). The glass bead/cell suspension mixture was stirred with a glass rod to remove trapped air. The bead-beater chamber was assembled, displacing as much air as  
20 possible from the chamber. The cells were ruptured by 2x1 minute pulses of the bead beater, separated by 4 minutes cooling between pulses. The lysate was transferred by decanting (to remove glass beads) into a pre-cooled 250ml Erlenmeyer flask. Fresh PMSF and pepstatin were added, and  
25 the lysate was transferred to 50ml screw-cap centrifuge tubes and centrifuged at 10,000Xg for 20 minutes to remove unbroken cells, cell wall fragments and mitochondria. The supernatant was centrifuged at 100,000xg for 60 minutes (Beckman Ultra-Clear 25x89mm; using the SW-28 rotor) and decanted into a  
30 pre-chilled 250ml Erlenmeyer Flask. Solid ammonium sulfate was gradually added to the high-speed supernatant to 40% saturation (24.2g/100ml). This mixture was left for 30 minutes at 4°C to equilibrate, with occasional gentle

swirling. The ammonium sulfate precipitate was collected by centrifugation at 15,000Xg for 30 minutes, discarding the supernatant. The resulting pellets can be left at 4°C overnight in the centrifuge tubes for resuspension the  
5 following day (ensure pellets do not dry out by adding 5ml Buffer A containing 40% Saturated Ammonium Sulfate). It is, however, preferable to carry the protein through the column purification step without stopping.

The lysis procedure can be repeated twice in a day,  
10 enabling the processing of 2 fermenters worth of cells at once. Glass beads can be rinsed with 2% Micro™ cleaning solution and re-used as necessary.

(3) Column Chromatography on Pierce ImmunoPure™ Monomeric  
15 Avidin Gel

(a) Pretreatment and Equilibration of Column.

The column at room temperature. The gel was diluted to 33% v/v with dI H<sub>2</sub>O and the corresponding slurry was used to  
20 pour the column (convenient disposable columns and instructions were supplied with the resin). After allowing the gel to settle (the column was stoppered at the bottom during pouring) a plastic frit was gently layered on top of the bed using the appropriate size serum separator provided  
25 with the column. The column was equilibrated after pouring with 2x2CV (column volumes) phosphate buffered saline (made up using Pierce BupH™ PBS pack). In order to block all the tight biotin-binding sites in the resin, the column was washed with Biotin Blocking and Elution Buffer (3x1CV). This  
30 was followed with 3x2CV Regeneration Buffer, which removes biotin from the loose-binding sites. The column was washed with 2x2CV PBS for storage, and can be tested for binding

capacity using biotinylated BSA (preferably it should be regenerated after measurement).

A 5-ml column is sufficient to purify all the enzyme from two fermenters worth of cells. Monomeric avidin Gel is  
5 stored in PBS (33% v/v gel) at 4°C before use.

(b) Purification of biotin-containing proteins.

All operations were performed at 4°C. The ammonium sulfate precipitate was taken up in a minimum volume of  
10 Buffer B + protease inhibitors. A 5ml Potter-Elvehjem homogenizer was used to facilitate the resuspension of the pellets. After homogenization, the protein solution was centrifuged (100,000xg, 60 minutes) to remove any insoluble material. Ammonium sulfate precipitates from two fermenters  
15 worth of cells can be resuspended in about 48ml Buffer B, which was sufficient liquid to fill 4 ultracentrifuge tubes (16x89mm, SW41-Ti rotor). Biotinylated proteins were batch-loaded onto monomeric avidin gel by adding 15ml 33% gel slurry to the combined hi-speed supernatant, and gently  
20 shaking the mixture in 50ml Falcon tubes on a rotating shaker (60rpm) for 1.5 hours (4°C). The mixture was immediately poured into a 10ml column (supplied by Pierce with the gel). After allowing time for the gel to settle, the resin was resuspended in 1CV Buffer B by capping the column and gently  
25 shaking. It was then left at 4°C to settle out again. After 45 minutes, the gel has formed a bed and a plastic frit was gently placed on top of the column to prevent drying. The column was washed with about 5CV of Buffer B (until the flow-through was essentially devoid of protein, as judged by  
30 Bradford Assay). ~1CV 2mM biotin in buffer B was applied to the column and the column capped for 1 hour to allow for exchange of bound proteins with free biotin. Proteins were eluted with 2mM biotin in buffer B, monitoring for protein

with the Bradford assay. Fractions were collected until no more protein eluted, and pooled. Pooled fractions were dialyzed overnight against Storage Buffer (which contains 50% glycerol). Protein was stored at -20°C.

- 5 Each enzyme preparation was tested for activity in the radiometric assay described below. In addition, the minimum amount of enzyme required for acceptable signal to noise and linearity in the assay (over a 45-minute time period) was determined for each batch. Several batches can be pooled and  
10 realiquoted, but the enzyme was re-assayed under the same conditions that will be used in the screen to ensure signal to noise and linearity were in the acceptable range.

(A) High-throughput screen

15

(I) Running the screen.

Reagents and amounts are summarized in the following table. Screening is performed in accordance with the protocol described in detail below.

20

Reagent	Max(100%)	Min (0%)	50%	Test
	D1	D2	D3	
	D7	D8	D9	
		Volume		
		( l)		
20% DMSO	20	20	20	20
Diluted enzyme in Assay Buffer	60	0	60	60
Assay Buffer	0	60	0	0
5X Radioactive Substrate Buffer	20	20	20	20
Myristoyl CoA (3mM in DMSO)	0	0	1	0
Test compound*	0	0	0	5 M final conc.

\*Test Compound stock diluted in BuOH 1:10; 25  $\mu$ l dried in assay plate in hood prior to addition of assay buffer. Well contains 0.5 nmol of each test compound (20/well) in 2.5  $\mu$ l 100% DMSO.

5

Potency of inhibitors can be quantified with respect to positive (no enzyme; 100% inhibition) and negative (no inhibitor; 0% inhibition) controls. The following formula can be used:

10

$$\% \text{ Inhibition} = \{1 - [A_{450} - (\text{positive control})] / [(\text{negative control}) - (\text{positive control})]\} * 100$$

#### (B) Radiometric Assay for Acetyl CoA Carboxylase

15

##### CONCENTRATIONS OF ASSAY COMPONENTS:

Component	Stock Concentration	Final Concentration
HEPES, pH 7.5	83.33mM*	50mM
MgCl <sub>2</sub>	8.333mM*	5mM
DTT	3.33mM*	2mM
ATP	20mM <sup>†</sup>	4mM
Acetyl CoA	375 M <sup>†</sup>	75 M
NaHCO <sub>3</sub>	5mM <sup>†</sup>	1mM
NaH[ <sup>14</sup> C]O <sub>3</sub>	2mCi/ml	10 Ci/ml <sup>†</sup>
*in ACCase Assay Buffer		
<sup>†</sup> in 5X Substrate <sup>†</sup> 10 Ci/ mol Buffer		

20 20  $\mu$ l of 20% DMSO are dispensed into all wells of each microtiter plate using a Titertec™ multidrop. 1  $\mu$ l of 3mM Myristoyl CoA in DMSO is added to appropriate control wells (D3 and D9). 60  $\mu$ l enzyme (pre-diluted with ACCase Assay Buffer) are added to all wells by the Titertec™, covering



wells with adhesive foil for no-enzyme controls (D2 and D8). In the control wells, enzyme solution forms a bead on top of the foil, and does not splash into adjacent wells. After enzyme addition, the bead on top of the foil is removed with  
5 a KimWipe™ and the foil removed for subsequent additions.  
60 1 of ACCase Assay Buffer (no enzyme) are then added to wells D2 and D8.

The 5X Radiometric Substrate Buffer is prepared from 5X Cold Substrate Buffer by the addition of  $\text{NaH}^{14}\text{C}\text{O}_3$ .  
10 Immediately after radioactive bicarbonate is added, the assay reaction is initiated by addition of 20 l 5X radiometric substrate buffer to each well using a Titertec™ MultiDrop dispenser. The Titertec™ is rinsed with 2N HCl (stop solution) immediately after dispensing the substrate buffer  
15 in preparation for the termination step of the assay. The reaction is stopped after a 40 minute incubation period (room temperature), by the addition of 100 l Stop Solution (using the Titertec™).

The plates are dried overnight at 45°C on a Slide Warmer.  
20 10 plates can be laid out on each slide warmer. Using the Titertec™, 100 l dI H<sub>2</sub>O are added to the dried wells to resolubilize the product, and 80 l of this material are transferred to an OptiPlate™. The transfer step is accomplished using the Tomtec™ automatic dispenser. 150 l  
25 of MicroScint™40, are added by Titertec™ (dedicated dispensing head). The plates are counted (after 12 hours equilibration) using a Packard TopCount (count 60 seconds; 1 minute delay before reading plate). Data are saved to a file for analysis by Microsoft Excel™.

30

**Purification of ACCase from crude lysates**

<sup>14</sup>C-labeled bicarbonate and ammonium sulfate are obtained from ICN Biomedicals. All supplies and gels for polyacrylamide gel electrophoresis and streptavidin blots are  
5 purchased from Bio-Rad. All other fine chemicals are purchased from Sigma. Sepharose CL-4B is purchased from Pharmacia. Promega Soft-Link, Soft Release avidin affinity resin is purchased from Fisher Scientific.

A fungal lysate is prepared in 225 mM mannitol, 75 mM  
10 sucrose, 10 mM Tris/HCl pH 7.5, 0.05 mM EDTA, 5 mM potassium citrate, 2.5 mM MnCl<sub>2</sub>, 10 mg/l each of aprotinin, leupeptin and antitrypsin (buffer A) is filtered through glass wool and powdered ammonium sulfate is added slowly, with stirring, to 35% saturation (200 g ammonium sulfate/l). Following 45  
15 minutes of continued stirring, precipitated protein is collected by centrifugation (30 min, 17000 X g). The precipitate is gently resuspended with stirring in a minimum volume of 100 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 1.0 mM EDTA, 0.1 mM dithiothreitol and 10% glycerol (buffer B). Insoluble  
20 material is then removed by centrifugation (20 min, 40000 X g) and the clarified supernatant dialyzed for 3 hours against 150 vol. 100 mM Tris/HCl, 0.5 M NaCl, 1.0 mM EDTA, 0.1 mM dithiothreitol and 5% glycerol (buffer C) to remove citrate.

The dialyzed suspension (approximately 30 ml) is then shaken  
25 with 40 ml affinity-column material [prepared by the procedure of Beaty and Lane 1982 (J Biol Chem 257: 924-929) and 30 ml buffer C for 3 hours, rinsed in a fritted funnel using 2 liters buffer C with stirring, and packed into a column with continual rinsing (10 - 15 ml/hour) until a  
30 stable baseline is achieved. The effluent from the column is monitored at 280 nm using an ISCO UA-6 absorbance detector. Avidin-bound material is eluted with buffer C (0.2 mM in biotin) at 5 - 8 ml/hour.

**ACCCase Assay**

Fungal acetyl-CoA carboxylase is assayed by a modification of the  $^{14}\text{CO}_2$  fixation assay of Thampy and Wakil (1985 J Biol Chem 260: 6318-6323) as follows: Purified enzyme (0.05 g) is added to a reaction mix (50 mM Hepes, pH 7.5, 1.5 mM magnesium sulfate, 2.0 mM dithiothreitol, 0.25 mM acetyl-CoA, 4.0 mM ATP, 12.5 mM  $\text{KHCO}_3$  ( $2 \times 10^6$  cpm  $\text{NaH}[^{14}\text{C}]\text{O}_3$ ), 0.75 g/l fatty-acid-free bovine serum albumin, 20 mM sodium citrate, 20 mM magnesium acetate in a total volume of 200  $\mu\text{l}$ ) at  $37^\circ\text{C}$  for 2 min. Reactions are terminated by the addition of 50  $\mu\text{l}$  5 M HCl. Aliquots (200  $\mu\text{l}$ ) are transferred and evaporated to dryness ( $80^\circ\text{C}$  in a hood under a gentle air stream). After cooling, distilled water (400  $\mu\text{l}$ ) is added followed by the addition of 5.5 ml Scintiverse II (Fisher). Radioactivity is determined in a Beckman LS 6500 Scintillation system. Assay blanks consisted of a standard assay with HCl added prior to enzyme. Typical background activity averaged 50 cpm. One unit activity is equal to 1  $\mu\text{mol}$   $\text{NaH}[^{14}\text{C}]\text{O}_3$  incorporated into acid-stable material/minute at  $37^\circ\text{C}$ . The specific activity is given in units/mg protein.

**Protein determination**

Protein is determined using the Bio-Rad Protein assay dye kit using bovine serum albumin as a standard.

**Polyacrylamide gel electrophoresis**

Protein samples are prepared by diluting in 62.5 mM Tris, pH 6.8, 10% glycerol, 2.5% (mass/vol.) SDS, 5% (by vol.) 2-mercaptoethanol, 0.025% (mass/vol.) Bromphenol blue (SDS sample buffer), and heating ( $95^\circ\text{C}$ , 4 min). Polyacrylamide gel electrophoresis is performed with the Bio-Rad Mini-Protean II Dual Slab Vertical Electrophoresis System using

Mini-Protean II 4-15 % gradient precast gels (Bio-Rad). Gels are run in the presence of 0.1 % SDS, 25 mM Tris, 192 mM glycine, pH 8.3, at 200 V for 60 min.

#### 5 Gel staining

Pooled column fractions are run parallel to high-range molecular-mass standards (silver staining) and to biotinylated high-range molecular-mass standards (Western blotting). Both sets of standards are obtained from Bio-Rad.

- 10 Gels (run concurrently with those used for silver staining) are stained with silver staining according to established procedures. Reagents for silver staining are obtained in the form of a kit (Sigma).

#### 15 Western Blotting

Proteins are transferred to nitrocellulose (Mini Trans-Blot Electrophoretic Transfer system from Bio-Rad) as follows. Gels are sandwiched next to nitrocellulose membranes in buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) overnight using a cooling well (30 V, model 1000/500 power supply, Bio-Rad).

- 25 Biotin-containing proteins are detected by streptavidin-alkaline-phosphatase conjugate (Bio-Rad). Streptavidin-specific protein is visualized colorimetrically using color reagents supplied in a streptavidin-alkaline-phosphatase conjugate substrate kit obtained from Bio-Rad. Membranes are stored in distilled H<sub>2</sub>O.

#### Antibodies

- 30 Antibodies can be raised against ACCase enzyme of the present invention by conventional techniques.

#### Northern Hybridization and Probe preparation

Northern blots, obtained from Clontech (Clontech Laboratories, 1020 East Meadow Circle, Palo Alto, California, 94303, USA), are prehybridized for 1 hour in Expresshyb hybridization solution (Clontech - Clontech Laboratories, 1020 East Meadow Circle, Palo Alto, California, 94303, USA) at 55°C before a radiolabelled ACCase fragment (DNA is labelled using the Megaprime random labelling system (Amersham {Amersham place, Little Chalfont, Bucks, HP7 9NA UK}) strictly following the manufacturers instructions with 50 Ci of <sup>32</sup>P-dATP) is added to fresh Expresshyb and hybridized to the blot overnight at 55°C, with gentle shaking. Blots are then washed 3X at room temperature for 10 minutes each in 2 X SSC (150mM NaCl, 30mM Na.citrate) followed by 2 washes in 0.2 X SSC (15mM NaCl, 3mM Nacitrate) at 55°C for 20 minutes each. Blots are then exposed to autoradiographic film.

#### Polymerase Chain Reactions (PCR)

PCR reactions are performed using standard reagents and conditions. Briefly, all reaction buffers and enzymes are obtained in kit format from either Clontech {Clontech Laboratories, 1020 East Meadow Circle, Palo Alto, California, 94303, USA} (for rapid amplification of cDNA ends (RACE) reactions) or from Life Technologies (3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF UK) for standard PCR. Oligonucleotides are obtained from a commercial supplier (OSWEL DNA services, Lab 5005, Medical And Biological Sciences Building, University of Southampton, Bolderwood, Bassett Crescent East, Southampton, SO16 7PX UK) and used at a concentration of 400nM. Reactions are performed on a MJ Research PTC-200 thermal cycler, using cycling parameters as recommended by the manufacturer of the kit being used.

**Cloning of PCR products**

PCR derived DNA fragments are cloned using the TOPO cloning system (Cat No. K3001-0-1) supplied by Invitrogen (De Schelp  
5 12, 9351 NV Leek, The Netherlands), following the methods as outlined in the manufacturers method book and using the reagents as supplied.

10

Other Embodiments

It is to be understood that, while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by  
15 the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims. For example, other art-known assays to detect interactions of test compounds with proteins, or to detect inhibition of fungal growth also can be used with the ACCase  
20 gene. The invention also includes methods of making a pharmaceutical composition for use in inhibiting *A. fumigatus*. Specifically, the method includes formulating a pharmaceutically acceptable excipient with an antifungal agent, such as those described herein.

What is claimed is:

1. An isolated nucleic acid molecule selected from the group consisting of:
  - (a) a nucleic acid molecule that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:2, as depicted in Fig. 1;
  - (b) a nucleic acid molecule that encodes a polypeptide comprising at least 15 contiguous amino acids of SEQ ID NO:2; and
  - (c) a nucleic acid molecule that encodes a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:2, wherein the nucleic acid molecule hybridizes under stringent conditions to a nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO:1, as depicted in Fig. 1, or the complement of SEQ ID NO:1.
2. An isolated nucleic acid molecule selected from the group consisting of:
  - (a) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1;
  - (b) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1, wherein the "T"s are replaced with "U"s;
  - (c) a nucleic acid molecule that is complementary to (a) or (b); and
  - (d) fragments of (a), (b) or (c) that comprise at least

- 30 contiguous nucleotides of SEQ ID NO:1 or the complement of SEQ ID NO:1.
3. An isolated nucleic acid molecule selected from the group consisting of:
- (a) a nucleic acid molecule comprising a nucleotide sequence which is at least about 75% identical to the nucleotide sequence of SEQ ID NO:1, or a complement thereof wherein the percent identity is calculated using the GAP program in the GCG software package, using a gap weight of 5.000 and a length weight of 0.100;
  - (b) a nucleic acid molecule comprising a nucleotide sequence that hybridizes to a nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO:1 under stringent conditions, or a complement thereof; and
  - (c) a nucleic acid molecule comprising a nucleotide sequence that hybridizes under stringent conditions to a nucleic acid molecule consisting of the nucleotide sequence of the cDNA insert of a plasmid deposited with the ATCC as Accession Number 207005, 207006, 207007, 207008, or 207009, or a complement thereof.
4. A nucleic acid molecule of any one of claims 1, 2, or 3, further comprising a vector nucleic acid sequence.



5. A nucleic acid molecule of any one of claims 1, 2, or 3, further comprising a nucleic acid sequence encoding a heterologous polypeptide.
6. A host cell that contains the nucleic acid molecule of any one of claims 1, 2, or 3.
7. A host cell of claim 6, wherein the cell is a mammalian host cell.
8. A host cell of claim 6, wherein the cell is a non-mammalian host cell.
9. An isolated polypeptide selected from the group consisting of:
  - (a) a polypeptide comprising a sequence of at least 15 contiguous amino acids of SEQ ID NO:2;
  - (b) a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:2 or an amino acid sequence encoded by the cDNA insert of the plasmid deposited with ATCC as Accession Number 207005, 207006, 207007, 207008, or 207009, wherein the polypeptide is encoded by a nucleic acid molecule that hybridizes under stringent conditions to the complement of a nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO:1; and
  - (c) a polypeptide encoded by a nucleic acid molecule comprising a nucleotide sequence that is at least 75% identical to SEQ ID NO:1

wherein the percent identity is calculated using the GAP program in the GCG software package, using a gap weight of 5.000 and a length weight of 0.100.

10. A polypeptide of claim 9, further comprising a heterologous amino acid sequence.
11. An antibody that selectively binds to a polypeptide of claim 9.
12. A method for producing a polypeptide comprising culturing the host cell of claim 6 under conditions in which the nucleic acid molecule is expressed, wherein the polypeptide is selected from the group consisting of:
  - (a) a polypeptide comprising the amino acid sequence of SEQ ID NO:2;
  - (b) a polypeptide comprising at least 15 contiguous amino acids of SEQ ID NO:2; and
  - (c) a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:2, wherein the polypeptide is encoded by a nucleic acid molecule that hybridizes under stringent conditions to a nucleic acid molecule comprising SEQ ID NO:1;the method comprising culturing the host cell of claim 6 under conditions in which the nucleic acid molecule is expressed.

13. A method for identifying an antifungal agent, the method comprising:

- (a) preparing a first cell and a second cell, the first and second cells being capable of expressing afACC1;
- (b) contacting the first cell with a test compound;
- (c) determining the level of expression of afACC1 in the first and second cells;
- (d) comparing the level of expression in the first cell with the second cell;

and

(e) selecting the test compound as an antifungal agent for the treatment of the fungal infection where the expression of afACC1 in the first cell is less than the expression of afACC1 in the second cell, wherein afACC1 is a first nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:2 or a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:2, and wherein the first nucleic acid molecule hybridizes to a second nucleic acid molecule under stringent conditions, the second nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO: 1 or the complement of SEQ ID NO:1.

14. A method of claim 13, wherein the level of expression is measured by measuring the amount of afACC1 mRNA in the cell.

15. A method of claim 13, wherein the level of expression is measured by measuring the amount of protein encoded by afACC1.

16. A method for identifying an antifungal agent for the treatment of a fungal infection comprising the steps of:

- (a) preparing a first cell and a second cell, the first and second cells being capable of expressing afACC1;
- (b) contacting the first cell with a test compound;
- (c) determining the level of expression of afACC1 in the first and second cells;
- (d) comparing the level of expression in the first cell with the second cell;

and

- (e) selecting the test compound as an antifungal agent for the treatment of the fungal infection where the activity of the polypeptide encoded by afACC1 in the first cell is less than the activity of the polypeptide encoded by afACC1 in the second cell;

wherein afACC1 is a first nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:2 or a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:2, wherein the first nucleic acid molecule hybridizes to a second nucleic acid molecule under stringent conditions, the second nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO: 1 or the complement of SEQ ID NO:1.

17. A method for identifying an antifungal agent for the treatment of a fungal infection comprising the steps of:

- (a) preparing a first sample of cells and a second sample of cells, the first and second samples of cells being

capable of expressing afACC1 in the presence of a test compound;

(b) contacting the first sample of cells with a test compound;

(c) comparing the growth of the first sample of cells with the second sample of cells;

(d) selecting the test compound as an antifungal agent for the treatment of the fungal infection where the growth of the first sample of cells is slower than the growth of the second sample of cells;

wherein afACC1 is a first nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:2 or a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:2, wherein the first nucleic acid molecule hybridizes to a second nucleic acid molecule under stringent conditions, the second nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO: 1 or the complement of SEQ ID NO:1.

18. A method of claim 17, wherein the first and second samples of cells comprise fungal cells.

19. A method of treating a fungal infection in a patient, comprising administering to the patient an effective amount of an antifungal identified using any one of claims 13, 16, or 17.

20. A method of claim 19, wherein the compound is selected from the group consisting of polypeptides, ribonucleic acids, small molecules, and deoxyribonucleic acids.

21. A method of claim 19, wherein the compound is an antisense oligonucleotide.

22. A method of claim 19, wherein the compound is a ribozyme.

23. A method for identifying an antifungal agent useful for treating a fungal infection, the method comprising:

(a) contacting a polypeptide encoded by afACC1 with a test compound; and

(b) detecting binding of the test compound to the polypeptide, wherein a compound that binds to the afACC1 polypeptide is useful for treating a fungal infection, and wherein the polypeptide is encoded by a gene selected from the group consisting of a first nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:2 or a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:2, wherein the first nucleic acid molecule hybridizes to a second nucleic acid molecule under stringent conditions, the second nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO: 1 or the complement of SEQ ID NO:1, wherein binding indicates that the test compound is an antifungal agent.

24. A method of claim 23, further comprising:

determining whether the test compound that binds to the afACC1 polypeptide inhibits growth of fungi, relative to growth of fungi grown in the absence of the test compound, wherein

inhibition of growth indicates that the test compound is an antifungal agent.

25. A method of claim 23, wherein the test compound is immobilized on a substrate, and binding of the test compound to the afACC1 polypeptide is detected as immobilization of the afACC1 polypeptide on the immobilized test compound.

26. A method of claim 25, wherein immobilization of the afACC1 polypeptide on the test compound is detected in an immunoassay with an antibody that specifically binds to the afACC1 polypeptide.

27. A method of claim 23, wherein the test compound is selected from the group consisting of polypeptides, ribonucleic acids, small molecules, and deoxyribonucleic acids.

28. A method of claim 23, wherein:

the afACC1 polypeptide is provided as a first fusion protein comprising afACC1 polypeptide fused to (i) a transcription activation domain of a transcription factor or (ii) a DNA-binding domain of a transcription factor; and

the test compound is a polypeptide that is provided as a second fusion protein comprising the test compound fused to (i) a transcription activation domain of a transcription factor or (ii) a DNA-binding domain of a

transcription factor, to interact with the  
first fusion protein; and  
binding of the test compound to the afACC1  
polypeptide is detected as reconstitution of a  
transcription factor.

29. A pharmaceutical formulation for the treatment of a  
fungal infection comprising an antifungal agent identified  
by the method of claim 23, and a pharmaceutically  
acceptable excipient.

30. A method for treating an organism having a fungal  
infection, the method comprising administering to the  
organism a therapeutically effective amount of the  
pharmaceutical formulation of claim 29.

31. A method of claim 27, wherein the organism is a human.

32. A method of treating an antifungal infection in an  
organism, the method comprising administering to the  
organism a therapeutically effective amount of the antibody  
of claim 11.

33. A method of claim 32, wherein the antibody is a  
monoclonal antibody.

34. A pharmaceutical formulation for the treatment of a  
fungal infection in an organism comprising a ribozyme of  
claim 22 and a pharmaceutically acceptable excipient.



35. A pharmaceutical formulation for the treatment of a fungal infection in an organism comprising an antisense nucleic acid of claim 21 and a pharmaceutically acceptable excipient.

36. A method for identifying a compound useful for treating a fungal infection, the method comprising:

- (a) contacting a afACC1 polynucleotide with a test compound; and
- (b) detecting binding of the test compound to the afACC1 polynucleotide, wherein a compound that binds to the afACC1 polynucleotide is useful for treating a fungal infection, and wherein the afACC1 polynucleotide is selected from the group consisting of
  - (i) a nucleic acid molecule that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:2; and
  - (ii) a nucleic acid molecule that encodes a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO: 2, wherein the nucleic acid molecule hybridizes under stringent conditions to a nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO:1 or the complement of SEQ ID NO:1.

37. The method of claim 36, further comprising: determining whether a test compound that binds to the afACC1 polynucleotide inhibits growth of fungi, relative to

growth of fungi grown in the absence of the test compound wherein inhibition of growth indicates that the test compound is an antifungal compound.

38. The method of claim 36, wherein the test compound is selected from the group consisting of polypeptides, small molecules, ribonucleic acids, and deoxyribonucleic acids.

39. The method of claim 36, wherein the test compound is an antisense oligonucleotide.

40. The method of claim 36, wherein the test compound is a ribozyme.

41. A method for identifying a compound useful for treating a fungal infection, the method comprising:

- (a) contacting a homolog of afACC1 with a test compound; and
- (b) detecting binding of the test compound to the homolog of afACC1, is useful for treating a fungal infection, and wherein a compound that binds to the homolog of afACC1 wherein afACC1 is selected from the group consisting of a first nucleic acid molecule which encodes either a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:2, wherein the first nucleic acid molecule hybridizes to a second nucleic acid molecule under stringent conditions, the second nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO:1 or the complement of SEQ ID NO: 1.

42. A method of claim 41, further comprising:  
determining whether a test compound that binds to the homolog of afACC1 inhibits growth of fungi, relative to growth of fungi grown in the absence of a test compound that binds to the homolog of afACC1, wherein inhibition of growth indicates that the test compound is an antifungal agent.

43. A method of claim 41, wherein the homolog of afACC1 is derived from a non-pathogenic fungus.

44. A method of claim 41, wherein the homolog of afACC1 is derived from a pathogenic fungus.

45. A method of claim 41, wherein the test compound is immobilized on a substrate, and binding of the test compound to the homolog of afACC1 is detected as immobilization of the homolog of afACC1 on the immobilized test compound.

46. The method of claim 45, wherein immobilization of the homolog of afACC1 on the test compound is detected in an immunoassay with an antibody that specifically binds to the homolog of afACC1.

47. The method of claim 41, wherein the test compound is selected from the group consisting of polypeptides, ribonucleic acids, small molecules, and deoxyribonucleic acids.

48. A method for identifying a compound for the treatment of a fungal infection comprising the steps, in sequence, of:

- a) preparing a first cell and a second cell, the first and second cells being capable of expressing afACC1;
- b) contacting the first cell with a test compound;
- c) determining the level of expression of the afACC1 gene in the first and second cells;
- d) comparing the level of expression of the afACC1 gene in the first cell with the second cell; and
- e) selecting the test compound as being useful for treating a fungal infection where the expression of the afACC1 gene in the first cell is less than the level of expression of the afACC1 gene in the second cell,

wherein the afACC1 gene is a first nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 2, or a naturally occurring allelic variant thereof, and wherein the first nucleic acid molecule hybridizes under stringent conditions to a second nucleic acid molecule, the second nucleic acid molecule consisting of a nucleotide sequence of SEQ ID NO: 1.

49. A method for identifying a compound for the treatment of a fungal infection comprising the steps, in sequence, of:

- a) preparing a first cell and a second cell, the first and second cells being capable of expressing a homolog of afACC1;
- b) contacting the first cell with a test compound;
- c) determining the level of expression of the homolog of the afACC1 gene in the first and second cells;

d) comparing the level of expression of the homolog of the afACC1 gene in the first cell with the second cell; and  
e) selecting the test compound as being useful for treating a fungal infection where the expression of the homolog of the afACC1 gene in the first cell is less than the level of expression of the homolog of the afACC1 gene in the second cell,

wherein the homolog of afACC1 gene is a first nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 2, or a naturally occurring allelic variant thereof, and wherein the first nucleic acid molecule hybridizes under stringent conditions to a second nucleic acid molecule, the second nucleic acid molecule consisting of a nucleotide sequence of SEQ ID NO: 1.

1/5

(SEQ ID NO:1)

5'GATTTTATCATGAAGTTGGCTGGCAATGCCCGGCATCTGGAAGTCCA  
GCTGCTGGCTGATCAGTATGGAAACAACATCTCCCTGTTCCGGCAGAGA  
CTGCTCCGTACAGCGTCGTCACCAGAAGATTATCGAGGAGGCCCCCGT  
CACCATCGCCAAGCCCGCCACGTTCCAGGCCATGGAGCGTGCGGCTG  
TCAGCCTCGGAAAGCTGGTTGGTTACGTCTCCGCGGGTACCGTCGAGT  
ACCTGTACTCGCACGCCGATGACAAGTTCTACTTCCTCGAGTTGAACC  
CTCGTCTTCAGGTCGAGCACCCACCACCGAAATGGTCTCCGGTGTCA  
ACCTGCCAGCCGCCCAATTGCAAATCGCCATGGGTATCCCTCTGCACC  
GCATCCGTGACATCCGTCTGCTCTACGGGGTTGACCCCAACACCTCGT  
CCGAGATTGACTTCGACTTCTCCAGCGAAGAGAGCTTCAAAACGCAGC  
GCCGCCCTCAGCCCAAGGGACACACGACCGCCTGCCGTATCACTTCCG  
AAGATCCCGGCGAAGGTTTCAAGCCCTCCAGCGGTACCATGCATGAAT  
TGAAC TTCCGCAGTTCGTCCAACGTCTGGGGTTACTTCTCCGTCGGTA  
CCGCCGGTGGTATCCACAGCTTCTCCGACAGCCAGTTCGGTCACATCT  
TTGCCTACGGTGAGACGCGTTCGCGCTCGCGGAAACACATGGTTGTCTG  
CTCTGAAGGAGTTGAGCATCCGGGGTGACTTCCGCACGACAGTCGAG  
TACCTGATCAAGCTACTGGAGACCCCTGCTTTCGAGGATAACACCATC  
ACCACTGGATGGCTGGATCAGCTCATCTCGAACAAGCTGACCGCAGA  
GCGTCCGGATCCCATCGTGGCTGTTCTGTGCGGTGCGGTGACCAAGG  
CTCACCTGGCCAGCGAAGGCGGTGTGAGGAGTATCGCAAGGGCCTC  
GAAAAGGGTCAGGTGCCCTCCAATGACGTCCTCAAGACCGTCTTCCCC  
GTGGACTTCATCTACGAGGGCCAGCGGTACAAGTTCACCGCAACCAG  
AGCCGGCTTGACAGCTACCACCTGTTTCATCAACGGATCCAAGTGCTC  
GGTCGGTGTCCGTGCTCTGGCCGACGGTGGCCTGCTTGTGCTCCTGAA  
CGGTCTAGTCACAACGTCTACTGGAAGGAGGAAGCGGCTGCAACTC  
GCCTGAGTGTGACGGAAAGACGTGCTTGCTGGAGCAGGAGAATGAT  
CCTACTCAGCTTCGCTCCCCGTCCCCCGGAAAGCTTGTCAAGTTCACG  
GTCGAGAACGGCGAGCACGTCAAGGCTGGTCAGGCCTTCGCCGAAGT  
CGAGGTCATGAAGATG'TACATGCCCTGATTGCACAGGAAGATGGTA  
TTGTTACAGTCATCAAGCAGCCTGGTTCACCCCTCGAGGCCGGTGACA  
TCCTCGGTATTCTCGCTCTGGATGACCCATCTCGTGTACACATGCCC  
AGCCTTTTACCGGACAGCTGCCCGACCTTGGTCCCCCGCAAGTGGTCG  
GTAACAAGCCTCCTCAGAGATTCTCCCTCCTCCACAGTATTCTCGAGA  
ACATCCTCATGGGCTATGACAACCAAGTTATCATGAACACCACTCTGA  
AGGAGCTGGTTGAGGTTTTGCGGGATCCTGAACTTCCCTACGGTGAAT

FIG. 1A

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GGAACGCTCAGTCTTCTGCCCTTCATTCTCGTATGCCCCAGAAGCTGG  
ACGCTCAGCTTCAGAGCATCGTCGACAAGGCTCACGCCAGAAAGGCC  
GAGTTCCCCGCCAAGCAGCTGCAGAAGACTATCTCCCGCTTCATCGAG  
GAGAACGTCAACCCAGCCGACGCCGAGATCCTCAAAACCACTCTCCTC  
CCTCTTCAGCAGGTCATACCAAGTACATGGATGGCCTGAAGGCCAC  
GAGTTCAACGTCTTCGCTGGATTGCTGGAGCAGTACTACAAGGTCGAG  
AGCCTCTTCTCTGGCCGCAACATCCGCGACGAAGATGCCATCCTGAAG  
CTCAGAGAAGAGCACAAGGACGATATTGGCAGCGTCGTTCAAGCTGGT  
ACTGTCCACAGCCGTATTGGCGCGAAGAACAACCTCATTTTGGCCAT  
CCTGGCCATGTACCGCCCCAACCAGCCTGGTGCTGGCAATGTCGCAAA  
GTACTTCAAGCCCGTCCTGAAGAAGCTCACTGAACTTGAGTCGCGGCC  
CGCCGCCAAGGTCACCCTCAAGGCCCGTGAGGTCCTCATCCAGTGTGC  
GCTTCCCTCCATGGAGGAGCGTATGTCTCAGATGGAACCTATTCTGCG  
CTCCTCTGTTGTGCAATCCCGATACGGAGAGACCGGTTGGGACCACCG  
GGAGCCCGAATTCTCCGTCCTCAAGGAAGTGGTGGACTCCAAGTACAC  
CGTCTTCGACGTCCTGACCCGATTCTTCGTTTCATCCGGACCCTTGGGT  
CACCTGGCTGCTCTCGAGGTCTACATTGCGCGTGCCTACAGGGCCTA  
TACACTGAAGGGTATTCAGTACTACCCCGATGGAGAAGTCCCCCTGGT  
CTCCTGGGACTTTACGCTAGGCAAGCTTGGACAACCGGAGTTCGGTCC  
GTTCACTCCAACCAGATGTCTACCCAGCACACCTACTACGGAGTCCA  
ACCCCTTCAGAAGACTCAACTCCATTAGTGATATGTCATACCTGTCAA  
CGACAGCAGCAATGAGCCCCTCAGAAAGGGTGTCAATTGTTCCGGTTCA  
GTCCCTGGAAGACGCCGAGGAGCAGCTGCCTAAGGCCTTGGAGGCAC  
TCCCTCGTGCCGGGTGCAAGAGGAAGCCGGGCGAGAACGGGCTGATT  
GCAGACCTGAGGGCAAGCGTACCAGCCCCTCGCATTGAGTCGACAAT  
TGAATTGACCGGTGTCTGCAACGTGGCTGTCCGTGACCTCGAAGATCT  
TGACGACAACCAGATCGTTGCCAGATCAACACCATTCTTGCCGGCCT  
CAGGGACGAGTTGCTCGCTCGCCGCGTCCGCCGCGTGACCTTCATTG  
CGGCAAGGACGGCAGCTACCCTGGCTACTTCACCTTCCGTGGACCTAC  
CTACGAGGAAGATGAGAGCATCCGTACAGCGAACCTGCGCTCGCCT  
TCCAGCTTGAACCTCGGACGTCTGTCCAAATTCAAGATCAAGCCCGTCT  
TCACCGAGAACCAGGAACATCCACGTCTACGAGGCCATCGGCAAGGGC  
CCCGAGAACGACAACGCTGTGACAAGCGTTACTTCGTCCGTGCTGTG  
GTGCGCCCGGGCCGTCTCCGTGACGATATTCCCACCGCGGAGTACCTC  
ATCTCCGAGGCTGACCGTCTCATGAATGACATTCTGGATGCCCTGGAG  
ATCATCGGCAACAACAATTCTGATCCGAACCACATCTTCATCAACTTC  
TCGCCGGTGTTCAACCTGCAGCCCCAGGATGTGGAAGAGGCCTTGGC  
CGGTTTCCTTGAGCGCTTCGGTCGCCGTCTCTGGCGTCTCCGTGTAC  
CGGTGCCGAGATCCGTATTCTATGCACCGATCCTGCCACTGGCATGCC  
TTATCCTCTGCGTGTGATCATACCAACACCTACGGCTTCATCATCCA  
GGTTGAGCTGTGATTGAGAAGAAGTCCGAGAAGGGCGAGTGGCTCC  
TCCACAGCATCGGTGGTACCAACAAGCTCGGCTCGATGCACCTGCGTC

FIG. 1B

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CTGTCTCCACACCCTACCCGACCAAGGAGTGGCTTCAGCCCAAGCGTT  
ACAAGGCTCATGTTATGGGCACCCAATACGTCTACGATTTCCCCGAAT  
TGTTCCGACAGGCCTTCCAGAACAGCTGGGCCAAGGCTGTAGCCAAG  
ATCCCCTCCCTGGCCAGCAAGCGGCCCGCGGTTGGCGACTGCATTGA  
GTACAGCGAGCTTGTTCTCGATGATACCGACAACCTGATCGAAATCTC  
GAGAGGCCCAGGTACCAACACCCACGGTATGGTTGGATGGATCGTTA  
CCGCTCGCACCCCAGAGTATCCCGAAGGCCGACGGTTCATCATCGTTG  
CCAACGACATCACCTTCCAGATCGGTTCCCTTCGGTCCCCAGGAGGACA  
AGTTCTTCTACAAGTGTACCGAGTTGGCCAGGAAGCTTGGAATCCCTC  
GTATCTACCTCTCAGCCAACTCCGGTGCTCGC'ATCGGTATGGCCGAC  
GAGCTGATCCCCTACTTCTCCGTGGCTTGGAACGACCCCCAGAAGCCC  
GAGGCTGGATTCAAGTACCTTTACCTCACTCCCGAGGTCAAGCAAAAA  
TTCGATGCCAGTAAGAAGAAGGAGGTCATTACTGAGCTCATTACGAT  
GAGGGCGAAGAGCGCCACAAGATTACGACTATCATTGGTGCTAAAGA  
TGGCCTGGGTGTTGAGTGTCTGAAGGGCTCTGGCCTCATCGCCGGAG  
CTACCTCGCGCGCTTACGAGGACATCTTCACCATCACCTGGTCACT  
GCCGCTCCGTTGGTATTGGTGCTACCTTGTCGGTCTGGGCCAGAGAG  
CCATCCAAGTAGAAGGCCAGCCGATTATTCTGACTGGTGCCCCGGCCA  
TCAACAAGCTGTTGGGTGCGGAGGTTTACACATCTAACCTTCAGCTCG  
GTGGTACTCAGATCATGTACAAGAACGGTGTCTCTCACATGACTGCCA  
CCGATGACTTTGAGGGGTGTCAGAAGATTGTTGAGTGGATGTCTTTCG  
TTCCCGACAAGAAGGGTGCATCCATTCCCATCCTGCCCTGGTCCGATG  
ACTGGGACCCGCGATGTCGCCTACTACCCTCTTCTAAGCAGGCTTACG  
ATGTCCGCTGGCTCATCGCTGGTAAAAAGGATGAGGAAGGCTTCCTCC  
CTGGTCTGTTTCGATGCCGGATCCTTTGAGGAGGCTCTTGGTGGATGGG  
CTCGTACCGTTGTCGTTGGTTCGTCGCTCGCCTTGGTGGCATCCCTATGG  
GIGIAATTGCT'GTCGAGACTCGTTCGGTTGAGAACGTTACCCCTGCCG  
ACCCTGCCAACCCTGACTCCATGGAGGTGATCAGCCAGGAAGCCGGT  
GGTGTGTGGTACCCAAACTCGGCCTTCAAGACCGCTCAGGCCCTCCGC  
GACTTCAATAATGGCGAGCAGCTGCCCGTCATGATTCTGGCCAACTGG  
AGAGGCTTCTCCGGTGGCCAGCGTGACATGTACAACGAGGTTCTCAA  
GTACGGTTCCTACATCGTCGATGCTCTGGTCAAGTACGAGCAGCCCAT  
CTTCGTTTATATCCCACTTTTCGGTGAACCTTCGTGGTGGTTCATGGGTC  
GTCATTGATCCACGATCAACCCTGACCAGATGGAGATGTACGCTGAT  
GAGGAGGCTCGCGGTGGTGTCTCGAACCAGAAGGTATCGTGAACAT  
CAAGTACCGCCGTGAGAAGCAGCTCGACACTATGGCTCGTCTCGACG  
CCACGTACGGCGAGCTCCGTCTGTCTTGGAGACCCATCCCTCAGCA  
AGGAGCAGCTCTCAGAGATCAAGGCCAAGATGGCCGCTCGCGAAGAG  
CAGCTCCTcCCTGTCTACCTGCAGATCGCTCTGCAATTTGCTGATCTTC  
ACGACCGCGCTGGCCGCGATGGTGGCCAAGAATACCATCCGCAAGGCC  
CTGACCTGGAAGAACGCCAGACGCTTCTTCTACTGGCGTGTACGCCCG  
CGCTTGAGCgAGGAGCTCATTCTCAAGCGCATGGCCTCTGCcGCCCCG

FIG. 1C



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GCCGCCGTCTCCGGCGAGGCCACCGGCGCCATCCCTGCCACCGGACT  
CGTCGACGGCCAGACCCCATCCAATGAGAGCCCTCGCGCTAAGCACC  
TGCGCACCCCTGCACTCGTGGACCGGCTTCCTGGACGAGGAACTCGAG  
CAGACGACCGCAAGGTAGCCATGTGGTACGAGGAGAACAGAAAGGC  
CATCCAGATGAAGATCGAGGCCCTTAAGACCGACTCTGTGCGCCACCGA  
GATCGCCCAGCTGCTCATCAGCAACAAGGAGGGCGGTCTCAAGGGTG  
TGCAGCAAGTTCTCAGCATGCTGCCTGTGAGGAGAAGGAGCCCGGTG  
CTCAAGTACCTTGGGCTCACCATGAAAATAGAATGAAACATAGAACCG  
CCCCGGTGCATATCTTTTGTTCCCCCCCCCTTTACTGGATTATATTT  
TCCAAATTCCTGATCACATTACGGTACATTGAAGATGGCTTTATACAA  
GGCGGGTGCGTTAGGGTCTGTGTTTTGTTTGTCTGCACTACGGTTTTG  
CGTTTTTGTCTTGCATGGGTCTTGGAGGTTGCATCGGCTGATTACTATT  
GTATGCATTATGTTGGTATGCCTGATGTTCTCTGGCAATGTTTCTATTC  
ACTTTTTCTAGCAATGCCAATGAATCTTCGACTTCAAAAAAAAAAAAA  
AAAAA3'

- 1- biotin binding (bp 1342-1350)
- 2- carboxybiotin domain (bp 3982-4137)
- 3- acetyl coA binding (bp 4810-4929)
- TGA-STOP (bp 6046)

**FIG. 1D**

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(SEQ ID NO:2)

DFIMKLAGNARHLEVQLLADQYGNNISLFGRDCSVQRRHQKIIIEAPVTIAK  
PATFQAMERAASVLGKLVGYVSAGTVEYLYSHADDFYFLELNPRLOVEHP  
TTEMVSGVNLPAALQIAMGIPLHRJRDIRLLYGVDPNSSSEIDFDFSSEESFKT  
QRRPQPKGHTTACRITSEDPGEGFKPSSGTMHELNFRSSSNVWGYFSVGTAG  
GIHSFSDSQFGHIFAYGETRSASRKHMVVALKELSIRGDFRTTVEYLIKLETP  
AFEDNTTTTGWLDQLISNKLTAEPRDPIVAFLCGAVTKAHLASEGGVEEYRK  
GLEKGQVPSNDVLKTVFPVDFIYEGQRYKFTATRAGLDSYHLFINGSKCSVG  
VRALADGGLLVLLNGRSHNVYWKEEAAATRLSVDGKTCLEQENDPTQLRS  
PSPGKLVKFTVENGEHVKAGQAFAEVEVMKMYMPLIAQEDGIVQLIKQPGST  
LEAGDILGILALDDPSRVTHAQPF TGQLPDLGPPQVVG NKPPQRFSLHSILE  
NILMGYDNQVIMNTTLKELVEVLRDPPELPGEWNAQSSALHSRMPQKLD AQ  
LQSIVDKAHARKAEFPAKQLQKTISRFEIENVNPADAEILKTTLLPLQQVITKY  
MDGLKAHEFNVFAGLLEQYYKVESLFSGRNIRDEDAILKLREEHKDDIGSVV  
QLVLSHSRIGAKNNLILAILAMYRPNQPGAGNVAKYFKPV LKKL TELESRPA  
KVT LKAREVLIQCALPSMEERMSQMELILRSSVVESRYGETGWDHREPEFSVL  
KEVVDSKYTVFDVLTRFFVHPDPVWTLAALEVYIRRAYRAYTLKGIQYYPDG  
EVPLVSWDFTLGKLGQPEFGPFTPTRCLPQHTYYGVQPLQKTQLHZZYVIPV  
NDSSNEPLRKG VIVPVQSLEDAEEQLPKALEALPRAGSKRKPGENGLIADLRA  
SVPAPRIESTIELTGVCNVAVRDLEDLDDNQIVAQINTILAGLRDELLARRVR  
RVT FICGKDGSYPGYFTFRGPTYEEDESIRHSEPALAFQLELGRLSKFKIKPVFT  
ENRNIHVYEAIGKGPENDNAV D KRYFVRAVVRPGR LRDDIPTAEYLISEADR  
LMNDILDALEIIGNNNSDPNHIFINFSPVFNLPQDVEEALAGFLERFGRRLW  
RLRV TGAEIRILCTDPATGMPYPLRVITNTYGFIIQVELCIEKKSEKGEWLLHS  
IGGTNKLGSMLRVPSTPYPTKEWLQPKRYKAHVMTQYVYDFPELFRQAF  
QNSWAKAVAKIPSLASKRPAVGDCIEYSELVLDDTDNLIEISRGPGTNTHG MV  
GWIVTARTPEYPEGRRFIIVANDITFQIGSFGPQEDKFFYKCTELARKLGIPRIY  
LSANSGARIGMADELIPYFSVAWNDPQKPEAGFKYLYLTPEVKQKFDASKKK  
EVTTELHDEGEERHKITTIIGAKDGLGVECLKGSGLIAGATSRAYEDIFTITLV  
TCRSVGIGAYLVRLGQRAIQVEGQPIILTGAPAINKLLGREVYTSNLQLGGTQ  
IMYKNGVSHMTATDDFEGCQKIVEWMSFVPDKKGASIPLPWSDDWDPRCR  
LLPSSKQAYDVRWLIAGKKDEEGFLPGLFDAGSFEEALGGWARTVVVGRARL  
GGIPMGVIAVETRSENVTPADPANPDSMEVISQEAGGVWYPNSAFKTAQAL  
RDFNNGEQLPVMILANWRGFSGGQRDMYNEVLKYGSYIVDALVKYEQPIFV  
YIPPFGE LRGGSWVIDPTINPDQMEMYADEEARGGVLEPEGIVNIKYRREK  
QLDTMARLDATY GELRRRALEDPSLSKEQLSEIKAKMAAREEQLLPVYLQIAL  
QFADLHDRAGRMVAKNTIRKALTWKNARRFFYWRVRRRLSEELILKRMA SA  
APAAVSGEATGAIPATGLVDGQTPSNESPRAKHLRTLHSWTGFLDEELEHDD  
RKVAMWYEENRKA IQMKIEALKTDSVATEIAQLLISNKEGGLKGVQQVLSML  
PVRRRSPVLKYLGLTMKIE

FIG. 1E